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(57) Abstract A novel protein having the activity of suppressing the differentiation and/or maturation of osteoclasts and methods of the production of the same. This protein is produced from human fetal pulmonary fibroblasts and has a molecular weight of about 60 KD under reductive conditions or about 120 KD under nonreductive conditions. It can be isolated and purified from the culture medium of the above-mentioned cells. Alternatively, it can be produced by genetic engineering techniques. The invention also provides a cDNA for the genetic engineering production of the protein, an antibody showing an affinity specifically for the protein, and a method for assaying the protein with the use of this antibody.			

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Specification

Novel Protein and Methods for its Manufacture

Technological Field

The present invention concerns a novel protein showing activity of inhibiting osteoclast differentiation and/or maturation, referred to as osteoclastogenesis inhibitory factor (OCIF), and methods for its manufacture.

Prior Art

Human bone is constantly engaged in a repeated process of resorption and neoformation, and the cells which play a central role in this process are osteoblasts, which are in charge of bone formation, and osteoclasts, which are in charge of bone resorption. An example of a disease occurring due to abnormalities in the bone metabolism carried out by the cells is osteoporosis. This disease occurs when bone formation by osteoblasts is exceeded by bone resorption by osteoclasts. The mechanism of occurrence of this disease has yet to be fully clarified, but the disease causes bone pain and is a contributing factor in fractures due to brittleness of the bones. As the aging population increases, this disease, which causes elderly patients to be bedridden due to fractures, is becoming a major social problem, and there is an urgent need for therapeutic agents to treat the disease. It is expected that decreases in bone mass resulting from such bone metabolism abnormalities can be treated by inhibiting bone resorption, accelerating bone formation, or improving the balance between these two processes.

It is expected that bone formation can be accelerated by accelerating the proliferation, differentiation, and activation of the cells responsible for bone formation or by inhibiting the proliferation, differentiation, and activation of the cells responsible for bone resorption. In recent years, interest in the physiologically active proteins which have such an activity (the cytokines) has increased, and vigorous research has been pursued on these substances. Examples of cytokines which accelerate the proliferation or differentiation of osteoblasts include the fibroblast growth factor family (FGF; Rodan S.B. et al., *Endocrinology*, Vol. 121, pg. 1917, 1987), insulin-like growth factor-I (IGF-I; Hock J.M. et al., *Endocrinology*, Vol. 122, pg. 254, 1988), insulin-like growth

factor-II (IGF-II; McCarthy T. et al., *Endocrinology*, Vol. 124, pg. 301, 1989), activin A (Centrella M. et al., *Mol. Cell. Biol.*, Vol. 11, pg. 250, 1991), transforming growth factor- β (Noda M., *The Bone*, Vol. 2, pg. 29, 1988), vasculotropin (Varonique M. et al., *Biochem. Biophys. Res. Commun.*, Vol. 199, pg. 388, 1994) and the heterotopic bone-forming factor family (bone morphogenetic protein (BMP); BMP-II; Yamaguchi, A. et al., *J. Cell Biol.*, Vol. 113, pg. 682, 1991, OP-1; Sampath T.K. et al., *J. Biol. Chem.*, Vol. 267, pg. 20532, 1992, Knutsen R. et al., *Biochem. Biophys. Res. Commun.*, Vol. 194, pg. 1352, 1993).

On the other hand, examples of cytokines which have been reported to inhibit osteoclastogenesis, i.e., the differentiation and/or maturation of osteoclasts, include transforming growth factor- β (Chenu C. et al., *Proc. Natl. Acad. Sci. USA*, Vol. 85, pg. 5683, 1988) and interleukin-4 (Kasano K. et al., *Bone-Miner.*, Vol. 21, pg. 179, 1993). Moreover, examples of cytokines which have been reported to inhibit bone resorption by osteoclasts include calcitonin (*Bone-Miner.*, Vol. 17, pg. 347, 1992) macrophage colony-stimulating factor (Hattersley G. et al., *J. Cell. Physiol.*, Vol. 137, pg. 199, 1988), interleukin-4 (Watanabe K. et al., *Biochem. Biophys. Res. Commun.*, Vol. 172, pg. 1035, 1990), and interferon-gamma (Gowen M. et al., *J. Bone Miner. Res.*, Vol. 1, pg. 469, 1986).

It is expected that these cytokines will be used as agents for alleviating decreases in bone minerals due to their action of accelerating bone formation and inhibiting bone resorption, and clinical studies are being conducted on some of these cytokines, such as insulin-like growth factor-I and the heterotopic bone-forming family, as bone metabolism-improving agents. Furthermore, calcitonin is already on the market as a therapeutic agent and analgesic for the treatment of osteoporosis.

At the present time, agents such as active vitamin D₃, calcitonin and its derivatives, hormone preparations such as estradiol, ipriflavone, vitamin K₂ (menatetrione), and calcium preparations are being clinically used as agents for the treatment of diseases involving the bone in an effort to shorten said treatment. However, treatment methods using these drugs are not necessarily satisfactory in their efficacy or therapeutic results, and there is a need to develop new treatment methods to replace these. As we have mentioned in the preceding, bone metabolism is regulated by balancing bone formation and bone resorption, and the cytokines, which inhibit the differentiation and maturation of osteoclasts, are promising as therapeutic agents for the treatment of decreased bone mass in diseases such as osteoporosis.

The present invention was developed based on the above considerations, and its purpose is to provide a novel osteoclastogenesis inhibitory factor (OCIF) and effective methods for its manufacture. The inventors of the present invention conducted thorough research in view of the above-described situation and discovered OCIF, a protein having osteoclastogenesis inhibitory activity, i.e., an activity of inhibiting the differentiation/maturation of osteoclasts, in a culture solution of human fetal pulmonary fibroblast IMR-90 cells (submitted to the ATCC--submission receipt no. CCL 186).

Moreover, they also discovered that when pieces of alumina ceramic were used as a carrier for the cell culture, the osteoclastogenesis inhibitory factor of the present invention, OCIF, could be caused to accumulate in the culture medium in high concentrations and thus be effectively purified.

Furthermore, the inventors of the present invention established a method for effectively purifying the aforementioned protein OCIF by means of sequential treatment of the aforementioned culture solution on an ion-exchange column, an affinity column, and a reverse phase column with repeated adsorption and elution.

Next, the inventors of the present invention succeeded in cloning the cDNA coding for the natural-type OCIF protein obtained based on data on the amino acid sequence of said protein. Furthermore, the inventors of the present invention established a method for manufacturing a protein having the activity of inhibiting the differentiation and/or maturation of osteoclasts by means of genetic engineering techniques using this cDNA.

The present invention concerns a protein originating from human fetal pulmonary fibroblasts which has a molecular weight of approximately 60 kD in SDS-PAGE under reductive conditions and molecular weights of approximately 60 kD and approximately 120 kD in SDS-PAGE under non-reductive conditions, which shows an affinity for a cation exchanger and a heparin column, is characterized by showing reduced activity of inhibiting the differentiation/maturation of osteoclasts as a result of heat treatment for 10 minutes at 70°C or 30 minutes at 56°C, and loses its activity of inhibiting the differentiation/maturation of osteoclasts as a result of heat treatment for 10 minutes at 90°C. The amino acid sequence of the protein OCIF of the present invention is clearly different from that of existing osteoclastogenesis inhibitory factors.

Moreover, the present invention concerns a method for manufacturing the protein OCIF in which human fibroblasts are cultured, the culture solution is treated on a heparin column, the adsorbed fraction is eluted, this fraction is run on a cation-exchange column and adsorbed/eluted, and the aforementioned protein is then purified using an affinity column and a reverse phase column

and collected. The column treatment of the present invention does not consist simply of running culture solution, etc., on a heparin sepharose column, etc., but includes mixing of the culture solution with heparin sepharose, etc., by the batch method in order to obtain an identical effect to column treatment. Examples of the affinity column used in the present invention include the heparin column and the blue column. A particularly preferred example of the blue column is the Cibacron blue column. An example of the filler used in the Cibacron blue column is a substance obtained by binding to the pigment Cibacron blue F3GA using a hydrophilic synthetic polymer as a carrier, and such a column is referred to as an ordinary blue column.

Furthermore, the present invention also concerns a method for effectively manufacturing the aforementioned protein by using an alumina ceramic piece as a carrier and carrying out cell culturing.

The protein OCIF of the present invention can be isolated and purified in an efficient and high-yield manner from culture solution of human fibroblasts. Isolation and purification of the protein OCIF of the present invention from these raw materials can be carried out according to a commonly-used method of purification of proteinous substances from biosamples, and this may be carried out according to various purification procedures which make use of the physical and chemical properties of the protein OCIF. Examples of concentration means used in this connection include common biochemical treatment processes such as ultrafiltration, freeze-drying, and salting out. Moreover, means of purification which may be used include combinations of various procedures commonly used in the purification of proteinous substances, such as ion-exchange chromatography, affinity chromatography, gel filtration chromatography, hydrophobic chromatography, reverse phase chromatography, and electrophoresis for preparation use. It is particularly preferred to use human fetal pulmonary fibroblast IMR-90 cells (ATCC-CCL 186) as human fibroblasts used as raw materials. Furthermore, culturing of the human fetal pulmonary fibroblast IMR-90 cells used as raw materials may be carried out by causing these human fetal pulmonary fibroblast IMR-90 cells to adhere to an alumina ceramic piece, and culturing for approximately 1 week to 10 days in a stationary culture using a roller bottle with DMEM culture medium with 5% fresh bovine fetal serum added as a culture solution. Moreover, in carrying out purification treatment, it is preferable to add 0.1% CHAPS (3-[(3-cholamidopropyl)-dimethylammonio]-1-propane sulfonate as a surfactant.

The protein OCIF of the present invention can be obtained by first running a culture solution on a heparin column (heparin-sepharose CL-6B, Pharmacia Co.), carrying out elution with 10 mM tris-HCl buffer solution with a pH of 7.5, obtaining an OCIF fraction with heparin adsorption properties, running this fraction on a Q/anion-exchange column (HiLoad-Q/FF, Pharmacia Co.), and obtaining a basic OCIF fraction with heparin adsorption properties by collecting the non-adsorbed

fraction. The OCIF activity fraction obtained can be isolated and purified by running it on a S/anion-exchange column (HiLoad-S/HP, Pharmacia Co.), a heparin column (heparin-5PW, Tosoh Co.), a Cibacron blue column (Blue-5PW, Tosoh Co.), and a reverse phase column (BU-300 C4, Perkin-Elmer Co.), and this substance is specified according to the aforementioned properties.

Moreover, the present invention also concerns a method for cloning the cDNA coding for this protein based on the amino acid sequence of the natural-type OCIF protein obtained and using this cDNA to obtain the protein OCIF having an activity of inhibiting the differentiation and/or maturation of osteoclasts by means of genetic engineering techniques.

Specifically, after OCIF protein obtained according to the method of the present invention is treated with an endoprotease (such as lysyl endoprotease), the amino acid sequence of the peptide produced is determined, and a mixture of the oligonucleotides which can code for the internal amino acid sequence obtained is prepared.

Next, taking the prepared oligonucleotide mixture as a primer, the PCR method (and preferably, the RT-PCR method) is used to obtain an OCIFcDNA fragment. Taking this OCIFcDNA fragment as a probe, the entire cDNA of the OCIF is cloned from the cDNA library. The OCIFcDNA obtained is inserted into an expression vector to prepare an OCIF expression vector, and by introducing this vector into various cells and bacterial strains and causing it to be expressed, one can manufacture recombinant OCIF.

The present invention also relates to the novel proteins OCIF2, OCIF3, OCIF4, and OCIF5, which are analogs (variants) of the OCIF protein of the present invention having the aforementioned activity.

These analogs are obtained by hybridizing the cDNA library prepared using the poly (A)⁺RNA of IMR-90 cells with an OCIFcDNA fragment as a probe. The cDNA of these OCIF analogs can be inserted into an expression vector, and this OCIF analog expression vector can be manifested in an ordinary host and then purified by a common method to obtain the target analog protein.

Furthermore, the present invention also relates to OCIF mutants.

These mutants are obtained by converting a Cys residue which may play a role in the dimer formation of OCIF to a Ser residue, or by inserting a deletion mutation in natural-type OCIF. By means of the PCR method or cutting with a restriction enzyme, conversion of the OCIFcDNA may be carried out or a deletion mutation may be inserted. By inserting this cDNA into a vector having an appropriate expression promoter, transfecting it into eukaryotic cells such as mammalian cells, culturing these cells, and then carrying out purification from the culture solution by an ordinary method, one can obtain the target OCIF mutant.

Moreover, the present invention also relates to an anti-OCIF polyclonal antibody or to an OCIF assay method using said antibody.

Anti-OCIF polyclonal antibodies are prepared according to a common method using OCIF as an immunogen. As the antigen (immunogen) used in this case, natural-type OCIF obtained from IMR-90 culture solution, recombinant-type OCIF produced with microorganisms or eukaryotic cells as hosts using OCIFcDNA, a synthetic peptide designed based on the amino acid sequence of OCIF, or hydrolyzed partial peptides of OCIF may be used. Using these antigens, or if necessary, using immune adjuvants in combination, one can immunize an appropriate mammal and carry out purification from its serum according to a common method to obtain the anti-OCIF polyclonal antibody. By labeling the anti-OCIF polyclonal antibody obtained with an isotope or enzyme, it can be used in an assay system such as radioimmunoassay (IRA) or enzyme immunoassay. Using such assay systems, the OCIF concentration in biosamples such as blood and peritoneal fluid or cell culture solution can be easily assayed.

Moreover, the present invention also relates to anti-OCIF monoclonal antibodies and to an OCIF assay method using such antibodies.

Anti-OCIF monoclonal antibodies are prepared by a common method using OCIF as an immunogen. As antigens, one may use natural-type OCIF obtained from IMR-90 culture solution, gene recombination-type OCIF produced using microorganisms or eukaryotic cells as hosts, synthetic peptides designed based on the amino acid sequence of OCIF, or hydrolyzed partial peptides of OCIF. Using these antigens, one can carry out immunization of mammals, fuse cells immunized by the in vitro method with mammalian myeloma cells, etc., to produce a hybridoma, select clones from this hybridoma which produce antibodies recognizing OCIF, and culture these clones to produce the target antibodies. In preparing hybridomas, when mammals are used, one generally uses small animals such as mice or rats. Immunization is carried out by diluting OCIF to an appropriate concentration using physiological saline, etc., administering this solution intravenously or intraperitoneally, and as needed, concurrently administering immune adjuvant, with the animals being given 2-5 administrations at 2-20-day intervals. Animals immunized in this manner are subjected to necropsies, their spleens are extracted, and the spleen cells are used as immunocytes.

Examples of myelomas of murine origin which are subjected to cell fusion with immunocytes include P3/x63-Ag8, p3-U1, NS-1, MPC-11, SP-2/0, FO, P3x63Ag8, 653, and S194. Furthermore, examples of cell strains of rat origin include R-210. In the case of producing human-type antibodies, human B lymphocytes are immunized by the in vitro method, and by using human myeloma cells and cell strains transformed with EB virus as parent strains, one can obtain hybridomas which produce human-type antibodies.

Fusion of immunocytes and myeloma cell strains may be carried out by a commonly-known method, such as the method of Koehler and Milstein et al. (Koehler, G. et al., *Nature*, Vol. 256, 495-497, 1975), or the electric pulse method. The immunocytes and myeloma cell strain are mixed with the medium used in cell culturing (which does not contain FBS) at the usual cell count ratio, polyethylene glycol is added to carry out fusion treatment, and culturing can then be carried out using HAT selective culture medium to select the fused cells.

In sorting anti-OCIF antibody-producing strains, sorting may be carried out using any commonly-used antibody detection method, such as the ELISA method, the plaque method, the Ouchterlony method, or the agglutination method. Hybridomas sorted in this manner may be subjected to subculturing by a common culturing method, and may then be put in cold storage as needed. The hybridoma may be cultured according to a common method, or antibodies may be produced by transplantation into the peritoneal cavity of mammals. The antibodies may be purified by any common method, such as salting out, gel filtration, or affinity chromatography.

The antibodies obtained react specifically with OCIF and can be used in assaying and purification of OCIF. In the case of use for assaying of OCIF, one can label the antibodies with an isotope or enzyme and use them in an assay system such as radioimmunoassay (IRA) or enzyme immunoassay (EIA). In particular, as the antibodies obtained according to the present invention show differing antigen recognition sites, they have the characteristic of being suitable for use in the sandwich immunoassay method. By using this assay system, the concentration of OCIF in biosamples such as blood and the peritoneal fluid or cell culture solution can be easily assayed.

The activity of OCIF may be assayed by the method of Kumeiawa M. et al. (*Proteins/Nucleic Acids/Enzymes*, Vol. 34, pg. 999 (1989)) or that of Takahashi N. et al. (*Endocrinology*, Vol. 122, pg. 1373 (1988)). Specifically, using murine myeloma cells from 17-day-old mice as target cells, the inhibition of osteoclast formation in the presence of active vitamin D₃ (calcitriol) can be tested using inhibition of induction of tartaric acid-resistant acid phosphatase activity as an indicator.

Osteoclastogenesis inhibitory factor, or OCIF, which is the protein of the present invention, is useful as a pharmaceutical composition for treating and alleviating bone mass loss in osteoporosis, etc., bone metabolism abnormalities such as rheumatism and gonarthrosis, or bone metabolism abnormalities such as multiple myeloma, and it is also useful as an antigen for establishing the immunological diagnosis of these diseases. The protein of the present invention can be incorporated into a drug and administered either orally or parenterally. Specifically, preparations containing the protein of the present invention can be safely administered to humans and animals as pharmaceutical compositions containing as their active ingredient osteoclastogenesis inhibitory

factor, or OCIF.

Examples of possible forms of such pharmaceutical compositions include compositions for injection, compositions for intravenous drip infusion, suppositories, nasal preparations, sublingual preparations, and transdermally-absorbed preparations. In the case of compositions for injection, mixtures of a pharmacological effective dosage of the osteoclastogenesis inhibitory factor of the present invention and a pharmaceutically acceptable base is used, and such mixtures may include fillers/activating agents generally added to compositions for injection, such as amino acids, sugars, cellulose derivatives, and other organic/inorganic compounds. During preparation, moreover, injection preparations containing the osteoclastogenesis inhibitory factor of the present invention and these fillers/activating agents may be made into various preparation types according to a common method by adding, as needed, pH-adjusting agents, buffers, stabilizers, solubilizers, etc.

Simplified Explanation of the Figures

Fig. 1 shows the elution profiles obtained when crude purification product of a HiLoad-Q/FF non-absorbed fraction (Sample 3) was run on a HiLoad-S/HP column.

Fig. 2 shows the elution profiles obtained when heparin-5PW crude purification product (Sample 5) was run on a blue-5PW column.

Fig. 3 shows the elution profiles obtained when blue-5PW elution fractions 49-50 were run on a reverse phase column. Fig. 4 shows the results of SDS-PAGE of the final purified products under reductive and non-reductive conditions.

Explanation of Symbols

Lanes 1, 4: Molecular weight markers

Lanes 2, 5: Peak 6

Lanes 3, 6: Peak 7

Fig. 5 shows the elution profiles obtained when, following reductive pyridyl ethylation, lysyl endoprotease-treated peak 7 was run on a reverse phase column.

Fig. 6 shows the results of SDS-PAGE of natural-type (n) and recombinant-type (r) OCIF under non-reductive conditions. Moreover, (E) indicates the product obtained using 293/EBNA cells, and (C) indicates that obtained using CHO cells.

Explanation of symbols

Lane 1: Molecular weight markers

Lane 2: Monomer-type nOCIF

Lane 3: Dimer-type nOCIF

Lane 4: Monomer-type rOCIF (E)

Lane 5: Dimer-type rOCIF (E)

Lane 6: Monomer-type rOCIF (C)

Lane 7: Dimer-type rOCIF (C)

Fig. 7 shows the results of SDS-PAGE of natural-type (n) and recombinant-type (r) OCIF under reductive conditions. (E) indicates the product obtained using 293/EBNA cells, and (C) indicates that obtained using CHO cells.

Explanation of Symbols

Lane 8: Molecular weight markers

Lane 9: Monomer-type nOCIF

Lane 10: Dimer-type nOCIF

Lane 11: Monomer-type rOCIF (E)

Lane 12: Dimer-type rOCIF (E)

Lane 13: Monomer-type rOCIF (C)

Lane 14: Dimer-type rOCIF (C)

Fig. 8 shows the results of SDS-PAGE of natural-type (n) and recombinant-type (r) OCIF with the N-bond type sugar chain removed under reductive conditions. Moreover, (E) indicates the product obtained using 293/EBNA cells, and (C) indicates that obtained using CHO cells.

Explanation of symbols

Lane 15: Molecular weight marker

Lane 16: Monomer-type nOCIF

Lane 17: Dimer-type nOCIF

Lane 18: Monomer-type rOCIF (E)

Lane 19: Dimer-type rOCIF (E)

Lane 20: Monomer-type rOCIF (C)

Lane 21: Dimer-type rOCIF (C)

Fig. 9 shows a comparison of the amino acid sequences of OCIF and OCIF2.

Fig. 10 shows a comparison of the amino acid sequences of OCIF and OCIF3.

Fig. 11 shows a comparison of the amino acid sequences of OCIF and OCIF4.

Fig. 12 shows a comparison of the amino acid sequences of OCIF and OCIF5.

Fig. 13 shows a calibration curve of OCIF when anti-OCIF polyclonal antibodies were used.

Fig. 14 shows a calibration curve of OCIF when anti-OCIF monoclonal antibodies were used.

Fig. 15 shows the therapeutic effect of OCIF on osteoporosis.

Optimum Embodiment of the Invention

The following is an explanation of the

present invention in greater detail by means of practical examples. However, these examples are given by way of illustration only, and the present invention is not limited to them.

[Practical Example 1]

Preparation of human fibroblast IMR-90 culture solution

Human fetal pulmonary fibroblast IMR-90 cells (ATCC-CCL 186) were caused to adhere to an 80 g alumina ceramic piece (alumina 99.5%, Toshiba Ceramic Co.) in a roller bottle (490 cm², 110 x 171 mm, Corning Co.) and cultured. In culturing, 60 roller bottles were used, with 500 ml of DMEM culture medium with HEPES buffer solution containing 5% calf serum added (Gibco BRL Co.) being used for each roller bottle, and stationary culturing was carried out for a period of 7-10 days under conditions of 37°C and 5% CO₂. After culturing, the culture solution was recovered, and by adding fresh culture medium and carrying out culturing once, 30 l of IMR-90 culture solution was obtained. The culture solution obtained was taken as Sample 1.

[Practical Example 2]

Method of assaying osteoclastogenesis inhibitory activity

Assaying of the activity of the proteinous osteoclastogenesis inhibitory factor of the present invention was carried out according to the method of Kumeoka M. et al. (Proteins/Nucleic Acids/Enzymes, Vol. 34, pg. 999 (1989) and the method of Takahashi N. et al. (Endocrinology, Vol. 122, pg. 1373 (1988)). Specifically, using myelocytes isolated from mice approximately 17 days old, osteoclastogenesis testing was carried out in the presence of active vitamin D₃ by using induction of tartaric acid-resistant acid phosphatase activity as an indicator and measuring this inhibitory activity. Specifically, 100 µl of the sample diluted with 2 x 10⁻⁸ M of active vitamin D₃ and α-MEM culture medium containing 10% fetal bovine serum (Gibco BRL Co.) was placed in a 96-well microplate, 3 x 10⁵ myelocytes obtained from mice approximately 17 days old were suspended in α-MEM culture medium containing 10% fetal bovine serum and seeding was carried out, and culturing was then conducted for a period of one week in a 5% CO₂ atmosphere at a temperature of 37°C and humidity of 100%. On days 3 and 5 of culturing, 160 µl of the culture solution was discarded, and 160 µl of a sample diluted with α-MEM culture medium containing 1 x 10⁻⁸ M active vitamin D₃ and 10% fetal bovine serum was added. After 7 days of culturing, washing was carried

out using phosphate-buffered physiological saline, the cells were fixed for one minute at room temperature using an ethanol/acetone (1 : 1) solution, and osteoclastogenesis was detected by staining using an acid phosphatase activity measurement kit (Acid Phosphatase, Leukocyte, Catalog No. 387-A, Sigma Co.). The decrease in the number of cells positive for acid phosphatase activity in the presence of tartaric acid was taken as OCIF activity.

[Practical Example 3]

Purification of OCIF

i) Purification using heparin/sepharose CL-6B

After approximately 90 l of IMR-90 culture solution (Sample 1) was filtered using a 0.22 μ m filter (Hydrophilic Millidisk, 2,000 cm^2 , Millipore Co.), and the solution was then run three times on a heparin/sepharose CL-6B column (5 x 4.1 cm, gel content 80 ml) balanced with 10 mM tris-HCl buffer solution (abbreviated in the following as tris-HCl) containing 0.3 M NaCl, pH 7.5. After washing at a flow rate of 500 ml/hour with 10 mM tris-HCl at a pH of 7.5, elution was carried out at a pH of 7.5 with 10 mM tris-HCl containing 2 M NaCl, 900 ml of heparin/sepharose CL-6B adsorbed fraction was obtained, and the fraction obtained was taken as Sample 2.

ii) Purification using the HiLoad-Q/FF

After the heparin/sepharose adsorbed fraction (Sample 2) was dialyzed with respect to 10 mM tris-HCl at a pH of 7.5, CHAPS was added to a concentration of 0.1% and the mixture was left standing overnight at 4°C, and this mixture was then run twice on an anion-exchange column (HiLoad-Q/FF, 2.6 x 7 cm, Pharmacia Co.) balanced with 50 mM tris-HCl containing 0.1% CHAPS, pH 7.5, and 1,000 ml of non-adsorbed fraction was obtained. The fraction obtained was taken as Sample 3.

iii) Purification using the HiLoad-S/HP

The HiLoad-Q non-adsorbed fraction (Sample 3) was run on a cation-exchange column (HiLoad-S/HP, 2.6 x 10 cm, Pharmacia Co.) balanced using 50 mM tris-HCl containing 0.1% CHAPS, pH 7.5. After washing at a pH of 7.5 using 50 mM tris-HCl containing 0.1% CHAPS, elution was carried out for 100 minutes with a linear gradient to 1 M NaCl and a flow rate of 8 ml/min., and the mixture was divided into 12 ml fractions. Fractions 1-40 were divided into four groups of 10 fractions each, and using 100 μ l each, OCIF activity was assayed. OCIF activity was observed in fractions 11-30 (Fig. 1: In the figure, ++ indicates osteoclastogenesis inhibitory activity of 80% or more, + indicates inhibitory activity of 30-80%, and - indicates that activity was not detected). Fractions 21-30, which had higher specific activity, were taken as Sample 4.

iv) Purification using an affinity column (heparin-5PW)

After 120 ml of Sample 4 was diluted with 50 mM tris-HCl containing 240 ml of 0.1%

CHAPS, pH 7.5, it was run on an affinity column (heparin-5PW, 0.8 x 7.5 cm, Tosoh Co.) balanced with 50 mM tris-HCl containing 0.1% CHAPS, pH 7.5. After washing with 50 mM tris-HCl containing 0.1% CHAPS, pH 7.5, elution was carried out for 60 minutes with a linear gradient to 2 M NaCl at a flow rate of 0.5 ml/min., and the sample was separated into 0.5 ml fractions. Using 50 µl of the various fractions, OCIF activity was assayed, and 10 ml of an OCIF activity fraction eluted at approximately 0.7-1.3 M NaCl was taken as Sample 5.

v) Purification using an affinity column (blue-5PW)

After 10 ml of Sample 5 was washed with 50 mM tris-HCl containing 190 ml of 0.1% CHAPS, pH 7.5, it was run on an affinity column (blue-5PW, 0.5 x 5.0 cm, Tosoh Co.) balanced with 50 mM tris-HCl containing 0.1% CHAPS, pH 7.5. After washing with 50 mM tris-HCl containing 0.1% CHAPS, pH 7.5, elution was carried out with a linear gradient to 2 M NaCl with a flow rate of 0.5 ml/min., and the sample was separated into 0.5 ml fractions. Using 25 µl of the various fractions, OCIF activity was assayed, and OCIF activity fractions 49-70, which were eluted at approximately 1.0-1.6 M NaCl, were obtained (Fig. 2: In the figure, ++ indicates osteoclastogenesis inhibitory activity of 80% or above, and + indicates inhibitory activity of 30-80%.

vi) Purification using a reverse phase column

After 10 µl of 25% TFA (trifluoroacetic acid) was added to the 49-50 ml of fractions obtained, the mixture was run on a reverse phase column (BU-300, C4, 2.1 x 220 mm, Perkin-Elmer Co.) balanced with 25% acetonitrile containing 0.1% TFA, elution was carried out for 60 minutes with a linear gradient to 55% acetonitrile at a flow rate of 0.2 ml/min., and the various peaks were separated (Fig. 3). Using 100 µl of the various peak fractions, OCIF activity was assayed, and concentration-dependent activity was detected in peak 6 and peak 7. The results are shown in Table 1.

Table 1. OCIF activity eluted from a reverse phase column

Dilution factor	1/40	1/20	1/360	1/1080
Peak 6	++	++	+	-
Peak 7	++	+	-	-

(In the table, ++ indicates osteoclastogenesis inhibitory activity of 80% or more, + indicates inhibitory activity of 30-80%, and - indicates that no activity was detected).

[Practical Example 4]

Molecular weight measurement of OCIF

Using 40 µl each of peaks 6 and 7, for which OCIF activity had been observed.

SDS-polyacrylamide gel electrophoresis was carried out under reductive and non-reductive conditions. Specifically, 20 μ l each of the various peak fractions was separated into two tubes and concentrated at reduced pressure, the concentrate was dissolved with 1.5 μ l of 10 mM tris-HCl containing 1 mM EDTA, 2.5% SDS, and 0.01% bromophenol blue, pH 8, the various fractions were left standing overnight at 37°C under non-reductive and reductive conditions (in the presence of 5% 2-mercaptoethanol), and 1 μ l portions were then subjected to SDS-polyacrylamide gel electrophoresis. Electrophoresis was carried out using 10-15% acrylamide gradient gel (Pharmacia Co.), and the electrophoresis unit used was the Phast System (Pharmacia Co.). The molecular weight markers used were phosphorylase b (94 kD), bovine serum albumin (67 kD), ovalbumin (43 kD), carbonic anhydrase (30 kD), trypsin inhibitor (20.0 kD), and α -lactalbumin (14.4 kD). After completion of electrophoresis, silver staining was carried out using the Phast Gel Silver Stain Kit (Pharmacia Co.). The results are shown in Fig. 4.

These results showed that with respect to peak 6, protein bands at approximately 60 kD were detected under reductive and non-reductive conditions. Moreover, with respect to peak 7, protein bands were detected at approximately 60 kD under reductive conditions and at approximately 120 kD under non-reductive conditions. Accordingly, it is thought that peak 7 is a homodimer of the protein of peak 6.

[Practical Example 5]

Thermostability test of OCIF

20 μ l each of samples composed of a mixture of blue-5PW fractions 51-52 was taken, and after 30 μ l of 10 mM phosphate-buffered physiological saline, pH 7.2, was added, thermal treatment was carried out for 10 minutes at 70°C and 90°C and for 30 minutes at 56°C. Using these samples, OCIF activity was assayed according to the method described under Practical Example 2. The results are shown in Table 2.

Table 2. Thermal stability of OCIF

Dilution factor	1/300	1/900	1/2700
Untreated ++	+	-	
70°C, 10 min.	+	-	-
56°C, 30 min.	+	-	-
90°C, 10 min.	-	-	-

(In the table, ++ indicates osteoclastogenesis inhibitory activity of 80% or more, + indicates inhibitory activity of 30-80%, and - indicates that activity was not detected.)

[Practical Example 6]

Determination of internal amino acid sequence

With respect to blue-5PW fractions 51-70, two fractions each were mixed to make 1 ml, and after 10 µl of 25% TFA was added to the various samples, portions of 1 ml each were run 10 times on a reverse phase column (BU-300, C4, 2.1 x 220 mm, Perkin-Elmer Co.) balanced with 25% acetonitrile containing 0.1% TFA, elution was carried out for 60 minutes with a linear gradient to 55% acetonitrile at a flow rate of 0.2 ml/min., and peaks 6 and 7 were collected. Taking a portion of the obtained peaks 6 and 7, N terminal amino acid sequence analysis was conducted using a protein sequencer (Procise, Model 494, Perkin-Elmer Co.), but analysis was impossible, indicating the possibility that the N terminals of these proteins might be blocked. For this reason, the internal amino acid sequences of these proteins were analyzed. Specifically, after peaks 6 and 7 were centrifuged and condensed, 50 µl of 0.5 M tris-HCl, pH 8.5, containing 100 µg of dithiothreitol, 10 mM EDTA, 7 M guanidine hydrochloride, and 1% CHAPS was added, the mixture was left standing for 4 hours at room temperature to carry out reduction, 0.2 µl of 4-vinylpyridine was added, and the mixture was then left standing overnight at room temperature in a dark place in order to carry out pyridyl ethylation. 1 µl of 25% TFA was added to these samples, they were run on a reverse phase column (BU-300, C4, 2.1 x 30 mm, Perkin-Elmer Co.) balanced with 20% acetonitrile containing 0.1% TFA, elution was carried out for 30 minutes with a linear gradient to 55% acetonitrile and a flow rate of 0.3 ml/min., and reduced pyridyl ethylated OCIF samples were obtained. The individual reduced pyridyl ethylated samples were centrifuged and concentrated, and after being dissolved with 25 µl of 0.1 M tris-HCl, pH 9, containing 8 M urea and 0.1% Tween 80, they were dissolved with 73 µl of 0.1 M tris-HCl, pH 9, 0.02 µg of AP1 (lycyl endoprotease, Wako Pure Chemical Industries Ltd.) was added, and the mixture was reacted at 37°C for 15 minutes. 1 µl of 25% TFA was added to the reaction solution, it was run on a reverse phase column (RP-300, C8, 2.1 x 220 mm, Perkin-Elmer Co.) balanced with 0.1% TFA, elution was carried out for 70 minutes with a linear gradient to 50% acetonitrile and a flow rate of 0.2 ml/min., and peptide fragments were obtained (Fig. 5). Concerning the peptide fragments obtained (P1-P3), amino acid sequence analysis was carried out using a protein sequencer. The results are shown in the Sequence Table, Sequence Nos. 1-3.

[Practical Example 7]

Determination of cDNA sequence

i) Isolation of poly(A)⁺ RNA from IMR-90 cells

The poly(A)⁺ RNA of IMR-90 cells was isolated according to the manual using a Phast Track mRNA Isolation Kit (Invitrogen Co.). Using this method, approximately 10 µg of poly(A)

* RNA was obtained from 1×10^8 IMR-90 cells.

ii) Preparation of mixed primer

Based on the amino acid sequence of previously-obtained peptides (Sequence Table, Sequence Nos. 2 and 3), the following two mixed primers were synthesized. Specifically, an oligonucleotide mixture (mixed primer, No. 2F) having all of the base sequences capable of coding for the amino acid sequence from position 6 (Gln) to position 12 (Leu) of peptide P2 (the peptide of Sequence No. 2) was synthesized. Moreover, a mixture (mixed primer, No. 3R) of the complementary oligonucleotides with respect to all of the amino acid sequences capable of coding for the amino acid sequence from position 6 (His) to position 12 (Lys) of peptide P3 (the peptide of Sequence No. 3) was synthesized. The amino acid sequences of the mixed primers used are shown in Table 3.

Table 3

[see pg. 17]

iii) Amplification of OCIFcDNA fragment by PCR

Taking 1 μ g of the poly(A) * RNA obtained in Practical Example 7-i) as a template and using the Superscript II cDNA Synthesis Kit (Gibco BRL Co.), single-stranded cDNA was synthesized according to the protocol of said company, and using this cDNA and the primer shown in Practical Example 7-ii), PCR was carried out and an OCIFcDNA fraction was obtained. The conditions are shown below.

10X Ex Taq buffer (Takara Shuzo)	5 μ l
2.5 mM dNTP	4 μ l
cDNA solution	1 μ l
Ex Taq (Takara Shuzo)	0.25 μ l
Distilled water	29.75 μ l
40 μ M primer No. 2F	5 μ l
40 μ M primer No. 3R	5 μ l

After the above solution was mixed in a microcentrifuge tube, PCR was carried out under the following conditions. After pretreatment for 3 minutes at 95°C, a reaction in the 3 stages of 30 seconds at 95°C, 30 seconds at 50°C, and 2 minutes at 70°C was repeated 30 times, and the temperature was then maintained at 70°C for 5 minutes. A portion of the reaction solution was subjected to agarose gel electrophoresis, and it was confirmed that a uniform DNA fragment of

approximately 400 bp had been obtained.

[Practical Example 8]

Cloning of OCIFcDNA fragment amplified by PCR and determination of base sequence

The OCIFcDNA fragment obtained under Practical Example 7-iii) was inserted into a plasmid pBluescript II SK⁻ (Stratagene Co.) according to the method of Marchuk, D. et al. (Nucleic Acid Res., Vol. 19, pg. 1154, 1991) using the DNA Ligation Kit Ver. 2 (Takara Shuzo), and transformation of *E. coli* DH5 α (Gibco BRL Co.) was carried out. The transformed strain obtained was caused to proliferate, and a plasmid containing an inserted OCIFcDNA fragment of approximately 400 bp was purified according to the usual method. This plasmid was designated pBSOCIF, and the base sequence of the OCIFcDNA inserted into this plasmid was determined using a Taq Dye Deoxy Terminator Cycle Sequencing Kit (Perkin-Elmer Co.). The size of this OCIFcDNA was 397 bp. In the amino acid sequence composed of 132 amino acids predicted based on this base sequence, the internal amino acid sequences of OCIF used in designing the mixed primer (Sequence Nos. 2 and 3 in the sequence table) could be found on the N terminal and C terminal sides respectively. Moreover, the internal amino acid sequence of OCIF (Sequence No. 1) could be found in this amino acid sequence composed of 132 amino acids. Based on the above results, it was confirmed that the cloned cDNA of 397 bp was the OCIFcDNA fragment.

[Practical Example 9]

Preparation of DNA probe

By carrying out PCR under the conditions of Practical Example 7-iii) with a plasmid having an OCIFcDNA fragment of 397 bp inserted prepared in Practical Example 8 as a template, this OCIFcDNA fragment was amplified. After an OCIFcDNA fragment of 397 bp was isolated by agarose gel electrophoresis, purification was carried out using the QIAEX Gel Extraction Kit (Qiagen Co.). This DNA was labeled with [α ³²P]dCTP using the Megaprimer DNA Labeling Kit (Amersham Co.), and it was used as a probe for screening of the entire length of OCIFcDNA.

[Practical Example 10]

Preparation of cDNA library

Taking the 2.5 μ g of the poly(A)⁺ RNA obtained in Practical Example 7-i) as a template, cDNA was synthesized using the Great Lengths cDNA Synthesis Kit (Crontech¹ Co.) according to said company's protocol using an oligo(dT) primer, an EcoRI-SalI-Not-I adapter was added, cDNA size fractionation was carried out, and following ethanol precipitation, it was dissolved in 10 μ l of TE buffer. 0.1 μ g of the obtained cDNA with an adapter added was inserted into 1 μ g of a

¹Spelling of company name uncertain. - Tr.

[lambda]ZAP express vector (Stratagene Co.) which had been cut in advance with EcoRI using T4DNA ligase. cDNA recombinant phage DNA solution obtained in this manner was subjected to an in vitro packaging reaction using the Gigapak Gold II (Stratagene Co.) to prepare a [lambda]ZAP express recombinant phage.

[Practical Example 11]

Screening of recombinant phages

The recombinant phage obtained in Practical Example 10 was infected for 15 minutes at 37°C with E. coli XL1-Blue MRF⁺ (Stratagene Co.), it was added to NZY culture medium containing 0.7% agar heated to 50°C, and it was then poured onto a NZY agar culture medium plate. It was cultured overnight at 37°C, and High Bond N (Pharmacia Co.) was allowed to adhere for approximately 30 minutes to the plate on which plaques had formed. After this filter was subjected to alkali denaturing according to the usual method, it was neutralized and immersed in 2XSSC solution, and the DNA was then fixed on the filter using the UV Crosslink (Stratagene Co.). The filter obtained was immersed in hybridization buffer (Pharmacia Co.) containing 100 µg/ml of salmon sperm DNA, and after pretreatment for 4 hours at 65°C, it was transferred to the aforementioned buffer to which the aforementioned thermally denatured DNA probe (2×10^5 cpm/ml) had been added and subjected to hybridization overnight at 65°C. After completion of the reaction, the filter was washed twice with 2XSSC and twice each with 0.1 XSSC and 0.1% SDS solution at 65°C for 10 minutes each. The several positive clones obtained were purified by being screened two more times. From among these, a substance having an insert of approximately 1.6 kb was used as follows. This purified filter was designated [lambda]OCIF. The purified [lambda]OCIF was infected with E. coli XL1-Blue MRF⁺ according to the protocol of the [lambda]ZAP Express Cloning Kit (Stratagene Co.), multiple infection was carried out using the helper phage ExAssist (Stratagene Co.), and after this supernatant was infected with E. coli XL0LR (Stratagene Co.), a kanamycin-resistant strain was collected in order to obtain a transformed strain having the plasmid pBKOCIF in which the aforementioned 1.6 kb insert was inserted into pBKCMV (Stratagene Co.). This transformed strain was taken as pBK/01F10, and it was submitted to the Bioengineering and Industrial Technology Research Institute of the Agency of Industrial Science and Technology, Ministry of International Trade and Industry under Submission Receipt No. FERM BP-5267 (transferred from the original deposit of FERM P-14998 in accordance with the Budapest Convention on October 25, 1995). The transformed strain having this plasmid was caused to proliferate, and the plasmid was purified according to the usual method.

[Practical Example 12]

Determination of cDNA base sequence coding for the entire amino acid sequence of OCIF

The base sequence of the OCIFcDNA obtained in Practical Example 11 was determined using a Taq Dye Deoxy Terminator Cycle Sequencing Kit (Perkin-Elmer Co.). The primers used were T3 and T7 primers (Stratagene Co.), as well as a synthetic primer designed based on the base sequence of OCIFcDNA, and this sequence is shown under Sequence Nos. 16-29 in the Sequence Table.

The determined base sequence of OCIF is shown in Sequence No. 6, and the amino acid sequence inferred from this sequence is shown in Sequence No. 5.

[Practical Example 13]

Production of recombinant OCIF using 293/EBNA cells

i) Preparation of OCIFcDNA expression plasmid

The plasmid pBKOCIF, containing the inserted OCIFcDNA of approximately 1.6 kb obtained in Practical Example 11, was digested with the restriction enzymes BamHI and XhoI, the OCIFcDNA was cut out, and after isolation by agarose gel electrophoresis, purification was carried out using the QIAEX Gel Extraction Kit (Qiagen Co.). This OCIFcDNA was inserted into the expression plasmid pCEP4 (Invitrogen Co.) digested in advance with the restriction enzymes BamHI and XhoI using the Ligation Kit Ver. 2 (Takara Shuzo), and transformation of E. coli DH5 α (Gibco BRL Co.) was carried out. The transformed strain obtained was caused to proliferate, and the expression plasmid pCEPOCIF with the OCIFcDNA inserted was purified using a Qiagen column (Qiagen Co.). After the OCIF expression plasmid pCEPOCIF was caused to precipitate using ethanol, it was dissolved in sterile distilled water and the following procedure was followed.

ii) Transient expression of OCIFcDNA and measurement of the activity thereof

Using the OCIF expression plasmid pCEPOCIF obtained in Practical Example 13-i) recombinant OCIF was expressed according to the method described below, and its activity was assayed. 8×10^5 293/EBNA cells (Invitrogen Co.) were seeded into the wells of a 6-well plate using IMDM culture medium (Gibco BRL Co.) containing 10% fetal bovine serum (Gibco BRL Co.), and on the next day, after the culture medium had been removed, the cells were washed with serum-free IMDM culture medium. In accordance with the protocol for the addition of the reagent for transfection lipofectamine (Gibco BRL Co.), after pCEPOCIF diluted in advance using OPTI-MEM culture medium (Gibco BRL Co.) and polyfectamine had been mixed, the mixture was added to the cells in the various wells. The amounts of pCEPOCIF and lipofectamine used were 3 μ g and 12 μ l respectively. After 38 hours, the culture medium was removed, 1 ml of fresh OPTI-MEM culture medium was added, and after an additional 30 minutes, the culture medium was recovered and taken as a sample for assaying OCIF activity. Assaying of OCIF activity was carried out as follows. Osteoclastogenesis from myelocytes of 17-day-old mice in the presence of active

vitamin D₃ was tested using induction of tartaric acid-resistant acid phosphatase activity as an index, the inhibitory activity was assayed, and this was taken as OCIF activity. Specifically, 100 μ l of the sample diluted with 2×10^{-8} M active vitamin D₃ and α -MEM culture medium containing 10% fetal bovine serum (Gibco BRL Co.) was placed in a 96-well microplate, 3×10^5 murine myelocytes taken from 17-day-old mice were suspended in α -MEM culture medium containing 10% fetal bovine serum and seeded, and culturing was carried out for 1 week in a 5% CO₂ atmosphere at 37°C and humidity of 100%. On days 3 and 5 of culturing, 160 μ l of the culture solution was discarded, and 160 μ l of the sample diluted with 1×10^{-8} M active vitamin D₃ and α -MEM culture medium containing 10% fetal bovine serum was added. After day 7 of culturing, washing was carried out with phosphate-buffered physiological saline, the cells were fixed at room temperature for 1 minute with ethanol/acetone (1 : 1) solution, and osteoclastogenesis was detected by means of staining using an acid phosphatase activity measurement kit (Acid Phosphatase, Leukocyte, Catalog No. 387-A, Sigma Co.). The decrease in the number of cells positive for acid phosphatase activity in the presence of tartaric acid was taken as OCIF activity. These results, as shown in Table 4, confirm that this culture solution has the same activity as natural-type OCIF obtained previously from IMR-90 culture solution.

Table 4. OCIF activity in culture solution expressed with 293/EBNA cells

Dilution	02 / 1	04 / 1	08 / 1	061 / 1	023 / 1	046 / 1	
1/1280							
OCIF							
Gene introduction	++	++	++	++	++	+	-
Vector introduction	-	-	-	-	-	-	-
Untreated	-	-	-	-	-	-	-

(In the table, ++ indicates osteoclastogenesis inhibitory activity of 80% or more, + indicates inhibitory activity of 30-80%, and - indicates that activity was not detected.)

iii) Purification of recombinant-type OCIF of 293/EBNA cell origin

The 293/EBNA cells mentioned under Practical Example 13-ii) were mass-cultured to obtain 1.8 l of culture solution, CHAPS was added to this to a concentration of 0.1%, and filtering was carried out with a 0.22 μ M filter (Sterivex GS, Millipore Co.), and the mixture was then run on a 50 ml heparin/sepharose CL-GB column (2.6 x 10 cm, Pharmacia Co.) balanced with 10 mM tris-HCl, pH 7.5. After washing with 10 mM tris-HCl containing 0.1% CHAPS, pH 7.5, elution

was carried out for 100 minutes with a linear gradient to 2 M NaCl and a flow rate of 4 ml/min., and the mixture was separated into 8 ml fractions. Using 150 μ l of the various fractions, OCIF activity was assayed according to the method of Practical Example 2, and 112 ml of an OCIF activity fraction eluted at approximately 0.6-1.2 M NaCl was obtained.

After 112 ml of the OCIF activity fraction obtained was diluted to 1,000 ml with 10 mM tris-HCl containing 0.1% CHAPS, pH 7.5, it was run on an affinity column (heparin-5PW, 0.8 x 7.5 cm, Tosoh Co.) balanced with 10 mM tris-HCl containing 0.1% CHAPS, pH 7.5. After washing with 10 mM tris-HCl containing 0.1% CHAPS, elution was carried out for 60 minutes with a linear gradient to 2 M NaCl and a flow rate of 0.5 ml/min., and the mixture was separated into 0.5 ml fractions.

Using 4 μ l each of the fractions obtained, SDS-polyacrylamide gel electrophoresis was carried out under reductive and non-reductive conditions according to the method of Practical Example 4. The results showed that as the only OCIF bands detected for fractions 30-32 were at approximately 60 kD under reductive conditions and approximately 60 kD and 120 kD under non-reductive conditions, fractions 30-32 were collected and taken as recombinant-type OCIF (rOCIF (E)) fractions of purified 293/EBNA cell origin. The results of protein determination by the Lowry method using BSA as a standard clearly showed that 1.5 ml of 535 μ g/ml rOCIF (E) was obtained.

[Practical Example 14]

Production of recombinant-type OCIF from CHO cells

i) Preparation of OCIF expression plasmid

The plasmid pBKOCIF containing inserted OCIFcDNA of approximately 1.6 kb obtained in Practical Example 11 was digested with the restriction enzymes SalI and EcoRV, an OCIFcDNA fragment of approximately 1.4 kb was cut out, and after isolation by agarose gel electrophoresis, purification was carried out using the QIAEX Gel Extraction Kit (Qiagen Co.). Moreover, after the expression vector pcDL-SR α 296 (Molecular and Cellular Biology, Vol. 8, pp. 466-472, 1988) was digested with the restriction enzymes PstI and KpnI and an expression vector DNA fragment of approximately 3.4 kb was isolated by agarose gel electrophoresis, purification was carried out using the QIAEX Gel Extraction Kit (Qiagen Co.). Using the DNA Branding Kit (Takara Shuzo), and the terminals of these purified OCIFcDNA fragments and expression vector DNA fragments were smoothed. Next, using the Ligation Kit Ver. 2 (Takara Shuzo), an OCIFcDNA fragment was inserted into the smoothed expression vector DNA fragment, conversion of E. coli DH5 α (Gibco BRL Co.) was carried out, and a transformed strain having OCIF expression plasmid pSR α OCIF was obtained.

ii) Preparation of expression plasmid

The transformed strain having OCIF expression plasmid pSR α OCIF obtained in Practical Example 13-i) and the mouse DHFR gene expression plasmid pBAddSV presented in WO92/01053 were caused to proliferate by the usual method, treated by the alkali method and the polyethylene glycol method according to the method of Maniatis et al. (Molecular Cloning, 2nd Edition), and purified by the cesium chloride density gradient centrifugation method.

iii) Acclimation of CHOdhFr₋ cells with protein-free culture medium

CHOdhFr cells (ATCC-CRL9096) subcultured with IMDM culture medium (Gibco BRL Co.) containing 10% fetal bovine serum (Gibco BRL Co.) were acclimated with the serum-free culture medium EXcell 301 (JRH Biosciences Co.), and they were then acclimated with the protein-free culture medium EX-Cell PF CHO (JRH Biosciences Co.).

iv) Introduction of OCIF expression plasmid and DHFR expression plasmid into CHOdhFr₋ cells

Using the OCIF expression plasmid pSR α OCIF prepared in Practical Example 14-ii) and the DHFR expression plasmid bBAddSV, the CHOdhFr cells prepared in Practical Example 14-iii) were transformed by the electroporation method described below. After 200 μ g of the pSR α OCIF plasmid and 20 μ g of the pBAddSV plasmid were dissolved in a sterile manner in 0.8 ml of IMDM culture medium (Gibco BRL Co.) containing 10% fetal bovine serum (Gibco BRL Co.), 2×10^7 CHOdhFr cells were floated using this 0.8 ml. This cell floatation solution was placed in a cuvette (Biorad Co.), and using a gene pulser (Biorad Co.), transformation was carried out by the electroporation method under conditions of 360 V and 960 μ F. After being subjected to electroporation, the cell floatation solution was transferred to a T flask for floating cell use (Sumitomo Bakelite Co.) containing 10 ml of EX-Cell PF CHO culture medium, and culturing was carried out for 2 days under CHO₂ incubation. Using the EX-Cell PF CHO culture medium, seeding was carried out into a 96-well microplate at a concentration of 5,000 cells/well, and culturing was carried out for approximately 2 weeks. As the EX-Cell PF CHO culture medium does not contain nucleic acids and this medium cannot be used for proliferation of the parent strain, CHOdhFr, only cell strains expressing DHFR were selected. As OCIF expression plasmid was used in an amount 10 times greater than the DHFR expression plasmid, the majority of the cell strains expressing DHFR also express OCIF. The cell strains showing high-OCIF activity in the culture supernatant were screened from the obtained strains expressing DHFR by means of the assay method shown in Practical Example 2. Concerning the obtained high-OCIF production strains, cell cloning was carried out by the limiting dilution method using EX-Cell PF CHO culture medium, and with respect to the clones obtained, the cell strains showing high-OCIF activity in the culture supernatant

were screened and the OCIF high-production clone 5561 was obtained.

v) Production of recombinant-type OCIF

In order to produce recombinant OCIF (rOCIF), transformed CHO cells (5561) were inoculated onto EX-Cell 301 culture medium 31 to a concentration of 1×10^5 cells/ml, and culturing was carried out for 4 to 5 days at 37°C using a spinner flask. When the cell concentration reached approximately 1×10^6 cells/ml, approximately 2.7 l of culture medium was collected. Approximately 2.7 l of EX-Cell 301 culture medium was added, and culturing was repeated. Using 3 spinner flasks, approximately 20 l of culture solution was then collected.

vi) Purification of recombinant-type OCIF of CHO cell origin

CHAPS was added to 1 l of the culture solution obtained in Practical Example 14-v) to a concentration of 0.1%, and after filtering with a 0.22 μ M filter (Sterivex GS, Millipore Co.), the mixture was run on a 50 ml heparin/sepharose FF column (2.6 x 10 cm, Pharmacia Co.) balanced with 10 mM tris-HCl, pH 7.5. After washing with 10 mM tris-HCl containing 0.1% CHAPS, pH 7.5, elution was carried out for 100 minutes with a linear gradient to 2 M NaCl and a flow rate of 4 ml/min., and the mixture was divided into 8 ml fractions. Using 150 μ l of the various fractions, OCIF activity was assayed according to the method of Practical Example 2, and 112 ml of an OCIF activity fraction eluting at approximately 0.6-1.2 M was obtained.

After 112 ml of the OCIF activity fraction obtained was diluted in 1,200 ml of 10 mM tris-HCl containing 0.1% CHAPS, pH 7.5, it was run on an affinity column (blue-5PW, 0.5 x 5 cm, Tosoh Co.) balanced with 10 mM tris-HCl containing 0.1% CHAPS, pH 7.5. After washing with 10 mM tris-HCl containing 0.1% CHAPS, pH 7.5, elution was carried out for 90 minutes with a linear gradient to 3 M NaCl and a flow rate of 0.5 ml/min., and the mixture was separated into 0.5 ml fractions.

Using 4 μ l each of the fractions obtained, SDS-polyacrylamide gel electrophoresis was carried out under reductive and non-reductive conditions according to the method of Practical Example 4. As the results showed that OCIF bands were only detected in fractions 30-38 at approximately 60 kD under reductive conditions and at approximately 60 kD and 120 kD under non-reductive conditions, fractions 30-38 were collected and taken as a recombinant-type OCIF (rOCIF (C)) fraction of purified CHO cell origin. It was clear that using the Lowry method with BSA as a standard, 4.5 ml of 113 μ g/ml rOCIF (C) was obtained.

[Practical Example 15]

N terminal structural analysis of recombinant-type OCIF

3 μ g of purified rOCIF (E) and rOCIF (C) were fixed on a polyvinylidene trifluoride (PVDF) film using a ProSpin (Perkin-Elmer Co.), and after washing with 20% methanol, the N

terminal amino acid sequence was analyzed using a protein sequencer (Procise, Model 492, Perkin-Elmer Co.). The results are shown in Sequence No. 7 of the Sequence Table.

The N terminal amino acids of rOCIF (E) and rOCIF (C) run from the Met which is the translation initiation point of the amino acid sequence shown in Sequence No. 5 of the Sequence Table to the Glu at position 22, and the 21 amino acids from Met to Gln clearly form a signal peptide. Moreover, the fact that the terminal amino acid sequence of natural-type OCIF purified and obtained from IMR-90 culture solution could not be analyzed is considered attributable to the fact that the Glu of the N terminal was converted into pyroglutamic acid during culturing or purification.

[Practical Example 16]

Physical properties of recombinant-type (r) OCIF and natural-type (n) OCIF

i) Inhibition of osteoclastogenesis induced by vitamin D₃ in a murine myelocyte system

100 µl of purified rOCIF (E) and nOCIF subjected to continuous 1/2 dilution from 250 ng/ml with α-MEM culture medium (Gibco BRL Co.) containing 2×10^{-8} M active vitamin D₃ and 10% fetal bovine serum was placed on a 96-well microplate. 3×10^5 murine myelocytes from 17-day-old mice were suspended in α-MEM culture solution containing 100 µl of 10% fetal bovine serum and seeded into the wells, and culturing was then carried out for one week in a 5% CO₂ atmosphere under conditions of 37°C and humidity of 100%. After 7 days of culturing, staining was carried out according to the method of Practical Example 2 using an acid phosphatase activity assay kit (Acid Phosphatase, Leukocyte, Catalog No. 387-A, Sigma Co.), and osteoclastogenesis was detected. The decrease in the number of acid phosphatase activity-positive cells in the presence of tartaric acid was taken as OCIF activity. The rate of decrease in acid phosphatase activity-positive cells was calculated by solubilizing the pigment of the stained cells and measuring its absorbance. Specifically, the cells were fixed, 100 µl of a mixed solution (1 : 1) of 0.1 N sodium hydroxide/dimethyl sulfoxide was added to the various stained wells, and thorough agitation was carried out. After the pigment had been thoroughly dissolved, absorbance was measured using a microplate reader (immunoreader NJ-2000, Intermed Co.) with a measurement wave length of 590 nm and a control wave length of 490 nm. Moreover, wells without vitamin D₃ added were used as blank wells during measurement of absorbance. The results are shown in Table 5 expressed as percentages, taking the absorbance value of the wells to which OCIF was not added as 100.

Table 5. Inhibition of osteoclastogenesis by OCIF in a murine myelocyte system (vitamin D₃)

OCIF concentration (ng/ml)	250	125	63	31	16	0
rOCIF (E)	0	0	3	62	80	100
nOCIF	0	0	27	27	75	100

As was the case for nOCIF, rOCIF (E) also showed dosage-dependent osteoclastogenesis inhibitory activity at concentrations of 16 ng/ml or above.

ii) Inhibition of osteoclastogenesis induced by vitamin D₃ in a co-culturing system with stroma cells and murine spleen cells

A study of osteoclastogenesis induced by vitamin D₃ in a co-culture system of stroma cells and murine spleen cells was conducted according to the method of Uda et al. (Endocrinology, Vol. 125, pp. 1805-1813, 1989). Specifically, 100 µl of purified rOCIF (E), rOCIF (C), and nOCIF continuously diluted with α-MEM culture medium (Gibco BRL Co.) containing 2×10^{-8} M active vitamin D₃, 2×10^{-7} M dexamethasone, and 10% fetal bovine serum was placed in a 96-well microplate. 5×10^3 cells of the murine bone marrow stroma cell strain ST2 (RIKEN Cell Bank-RCB0224) and 1×10^5 murine spleen cells taken from 8-week-old ddy mice were suspended in 100 µl of α-MEM culture medium containing 10% fetal bovine serum and seeded into the wells of the plate, and culturing was then carried out for 5 days in a 5% CO₂ atmosphere under conditions of 37°C and humidity of 100%. After 5 days of culturing, after washing was conducted using phosphate-buffered physiological saline, the cells were fixed for 1 minute at room temperature using a solution of ethanol/acetone (1 : 1), and osteoclastogenesis was detected by staining using an acid phosphatase activity measurement kit (Acid Phosphatase, Leukocyte, Catalog No. 387-A, Sigma Co.). The decrease in the number of cells positive for acid phosphatase activity in the presence of tartaric acid was taken as OCIF activity. Moreover, the rate of decrease in the number of cells positive for acid phosphatase activity was calculated according to the method shown in Practical Example 16-i) by causing the pigment of stained cells to dissolve. Table 6 shows the results of tests using rOCIF (E) and rOCIF (C), and Fig. 7 shows the results of tests conducted using rOCIF (E) and nOCIF.

Table 6. Osteoclastogenesis inhibition by OCIF in a co-culturing system of stroma cells and murine spleen cells

OCIF concentration (ng/ml)	50	25	13	6	0
rOCIF (E)	3	22	83	80	100
rOCIF (C)	13	19	70	96	100

Table 7. Osteoclastogenesis inhibition by OCIF in a co-culturing system of stroma cells and murine spleen cells

OCIF concentration (ng/ml)	250	63	16	0
rOCIF (E)	7	27	37	100

As was the case for nOCIF, rOCIF (E) and rOCIF (C) also showed dosage-dependent osteoclastogenesis-inhibiting activity at concentrations of 6-16 ng/ml or above.

iii) Inhibition of osteoclastogenesis induced by PTH

A study of osteoclastogenesis induced by PTH was conducted according to the method of Takahashi et al. (Endocrinology, Vol. 122, pp. 1373-1382, 1988). Specifically, 100 μ l of nOCIF and purified rOCIF (E) continuously diluted from 125 ng/ml with α -MEM culture medium (Sigma Co.) containing 2×10^{-8} MPTH and 10% fetal bovine serum was placed in a 96-well microplate. 3×10^5 murine spleen cells from 17-day-old mice cells were suspended in 100 μ l of α -MEM culture medium containing 10% fetal bovine serum, and culturing was carried out for a period of 5 days in a 5% CO₂ atmosphere under conditions of 37°C and humidity of 100%. After 5 days of culturing, after washing with phosphate-buffered physiological saline, the cells were fixed for 1 minute at room temperature using an ethanol/acetone (1 : 1) solution, and osteoclastogenesis was detected by staining using an acid phosphatase activity measurement kit (Acid Phosphatase, Leukocyte, Catalog No. 387-A, Sigma Co.). The decrease in the number of cells positive for acid phosphatase activity in the presence of tartaric acid was taken as OCIF activity. Moreover, the rate of decrease in the number of cells positive for acid phosphatase activity was calculated according to the method shown in Practical Example 16-i) by dissolving the pigment of stained cells. The results are shown in Table 8.

Table 8. Osteoclastogenesis inhibition by OCIF in a murine bone marrow cell system (PTH)

OCIF concentration (ng/ml)	125	63	31	16	8	0
rOCIF (E)	6	58	58	53	88	100
nOCIF	18	47	53	56	91	100

As was the case for nOCIF, rOCIF (E) also showed dosage-dependent osteoclastogenesis-inhibiting activity at concentrations of 16 ng/ml or above.

iv) Inhibition of osteoclastogenesis induced by IL-11

A study of osteoclastogenesis induced by IL-11 was conducted according to the method of Tamura et al. (Proc. Natl. Acad. Sci. USA, Vol. 90, pp. 11924-11928, 1993). Specifically, 100 μ l of nOCIF and purified rOCIF (E) diluted with α -MEM culture medium (manufactured by Gibco BRL Co.) containing 20 ng/ml of IL-11 and 10% fetal bovine serum was placed in a 96-well microplate. 5×10^5 of the prelipid cell strain MC3T3-G2/PA6 of murine newborn cranial origin

(RIKEN Cell Bank-RCB1127) and 1×10^5 spleen cells from 8-week-old ddy mice were suspended in 100 μ l of α -MEM culture medium containing 10% fetal bovine serum and seeded into the wells, and culturing was carried out for 5 days in a 5% CO₂ atmosphere under conditions of 37°C and humidity of 100%. After 5 days of culturing, after washing with phosphate-buffered physiological saline, the cells were fixed for 1 minute at room temperature with an ethanol/acetone (1 : 1) solution, and osteoclastogenesis was detected by staining using an acid phosphatase activity measurement kit (Acid Phosphatase, Leukocyte, Catalog No. 387-A, Sigma Co.). The number of cells positive for acid phosphatase activity in the presence of tartaric acid was counted, and the decrease therein was taken as OCIF activity. The results are shown in Table 9.

Table 9. Number of cells positive for acid phosphatase activity in the presence of tartaric acid induced by IL-11

Concentration (ng/ml)	500	125	31	7.8	2.0	0.5	0
nOCIF	0	0	1	4	13	49	31
rOCIF (E)	0	0	1	3	10	37	31

Both nOCIF and rOCIF (E) showed dosage-dependent activity of inhibiting osteoclastogenesis induced by IL-11 at concentrations of 2 ng/ml or above.

In a test system of osteoclastogenesis using these various target cells, OCIF clearly inhibited osteoclastogenesis by various osteoclastogenesis-inducing factors such as vitamin D₃, PTH, and IL-11 at virtually the same concentration. Accordingly, these results indicated that OCIF can be effectively used in the treatment of different types of bone mass loss induced by these various bone-resorption-accelerating substances.

[Practical Example 17]

Preparation of monomer-type and dimer-type OCIF samples

After adding 1/100 of the volume of 25% TFA (trifluoroacetic acid) to samples containing 100 μ g of rOCIF (E) and rOCIF (C) respectively, the mixture was run on a reverse phase column (Protein-RP, 2.0 x 250 mm, YMC Co.) balanced with 30% acetonitrile containing 0.1% TFA, elution was carried out for 50 minutes with a linear gradient to 55% acetonitrile and a flow rate of 0.2 ml/min., and the various OCIF peaks were separated. By freeze-drying the peak fractions obtained, monomer-type OCIF and dimer-type OCIF were obtained.

[Practical Example 18]

Molecular weight measurement of recombinant-type OCIF

Samples containing approximately 1 μ g each of monomer-type and dimer-type nOCIF

purified using a reverse phase column according to the method of Practical Example 3-vi) and monomer-type and dimer-type rOCIF purified according to the method of Practical Example 17 were concentrated at reduced pressure. Using these samples, SDS treatment, SDS-polyacrylamide electrophoresis, and silver staining were carried out according to the method of Practical Example 4. The results of electrophoresis carried out under non-reductive and reductive conditions are shown in Figs. 6 and 7 respectively.

These results show that under non-reductive conditions, protein bands of 60 kD were detected for both monomer-type samples, while protein bands of 120 kD were detected for both dimer-type samples. Furthermore, under reductive conditions, only protein bands at approximately 60 kD were detected for both samples. Accordingly, it was found that the molecular weights of the various monomer-type and dimer-types of nOCIF of IMR-90 cell origin, recombinant-type OCIF of 293/EBNA cell origin, and recombinant-type OCIF of CHO cell origin were virtually identical.

[Practical Example 19]

Removal of N bond-type sugar chains and measurement of molecular weights of natural-type OCIF of IMR-90 cell origin and recombinant-type OCIF

Samples containing approximately 5 µg each of monomer-type or dimer-type OCIF purified using a reverse-phase column according to the method of Practical Example 3-vi) and monomer-type and dimer-type rOCIF purified by the method of Practical Example 17 were concentrated under reduced pressure. 9.5 µl of 50 mM phosphate buffer solution, pH 8.6, with 100 mM 2-mercaptoethanol added was added to the samples and dissolved, 0.5 µl of 250 U/ml N-glycanase solution (Seikagaku Kogyo Co.) was added, and the mixture was left standing for 1 day at 37°C. 10 µl of 20 mM tris-HCl, pH 8.0, containing 2 mM MEDTA, 5% SDS, and 0.02% bromophenol blue was added to these samples, and the mixture was heated at 100°C for 5 minutes. After 1 µl of the various samples was subjected to SDS-polyacrylamide electrophoresis according to the method of Practical Example 4, silver staining was carried out. These results are shown in Fig. 8.

The results show that the molecular weights of OCIF protein with the N bond-type sugar chain removed by N-glycanase treatment under reductive conditions were approximately 40 kD in all cases. As the molecular weights under reductive conditions of the nOCIF of IMR-90 cell origin, rOCIF of 293/EBNA cell origin, and the rOCIF of CHO cell origin, in which the sugar chains were not removed, were all approximately 60 kD, this clearly shows that these types of OCIF are glycoproteins having an N bond-type sugar chain in their molecule.

[Practical Example 20]

Cloning and base sequence determination of OCIF analog (variant) cDNA

As shown in Practical Examples 10 and 11, a transformant strain having the plasmid pBKOCIF, in which OCIF cDNA was inserted into pBKCMV (Stratagene Co.), was obtained from one of several purified positive phages, but in this process, transformant strains were also obtained having plasmids containing inserts of different lengths from several other positive phages. The transformed strains having these plasmids were caused to proliferate, and the plasmids were purified according to the usual method. The base sequence of these types of insert DNA was determined using a Taq Dye Deoxy Terminator Cycle Sequencing Kit (Perkin-Elmer Co.). The primers used were T3 and T7 primers (Stratagene Co.) and a synthetic primer designed based on the base sequence of OCIFcDNA. In addition to the original type OCIF, there were a total of four types of OCIF variants (OCIF 2, 3, 4, and 5). The determined base sequence of OCIF2 cDNA is shown in Sequence No. 8, and the amino acid sequence inferred from this sequence is shown in Sequence No. 9. The determined base sequence of OCIF3cDNA is shown in Sequence No. 10, and the amino acid sequence inferred from this sequence is shown in Sequence No. 11. The determined base sequence of OCIF4 cDNA is shown in Sequence No. 12, and the amino acid sequence inferred from this sequence is shown in Sequence No. 13. The determined base sequence of OCIF5 cDNA shown in Sequence No. 14, and the amino acid sequence inferred from this sequence is shown in Sequence No. 15. The structural characteristics of these OCIF variants are shown in Figs. 9-12 and simply explained in the following description.

OCIF2

There is a deletion of 21 bp from the guanine at position 265 to the guanine at position 285 of the base sequence of OCIFcDNA (Sequence No. 6), and in the amino acid sequence, there is a deletion of 7 amino acids from the glutamine (Glu) at position 68 to the glutamine (Gln) at position 74 of the amino acid sequence of OCIF (Sequence No. 5 in the Sequence Table).

OCIF3

The cytidine at position 9 of the base sequence of OCIFcDNA (Sequence No. 6) is converted to guanine, and in the amino acid sequence, the asparagine (Asn) at position -19 of the amino acid sequence of OCIF (Sequence No. 5 of the Sequence Table) is changed into lysine (Lys). However, this is amino acid conversion in the signal sequence, and it is not thought to have any effect on secreted OCIF3.

There is a deletion of 39 amino acids from 117 bp from the guanine at position 872 to the guanine at position 989 of the base sequence of OCIFcDNA (Sequence No. 6), and in the amino acid sequence, there is a deletion from the threonine (Thr) at position 270 to the leucine (Leu) at position 308 of the amino acid sequence of OCIF (Sequence No. 5 in the Sequence Table).

OCIF4

The cytidine at position 9 of the base sequence of OCIFcDNA (Sequence No. 6) is converted to guanine, and in the amino acid sequence, the asparagine (Asn) at position -19 of the amino acid sequence of OCIF (Sequence No. 5 in the Sequence Table) is changed into lysine (Lys). Moreover, the guanine at position 22 is converted into thymidine, and in the amino acid sequence, the alanine (Ala) at position -14 of the amino acid sequence of OCIF (Sequence No. 5 in the Sequence Table) is changed into serine. However, these are amino acid conversions in the signal sequence, and they are not thought to have any effect on secreted OCIF4.

An intron 2 of approximately 4 kb is inserted between position 400 and position 401 of the base sequence of OCIFcDNA (Sequence No. 6), and the open reading frame ends in this intron. In the amino acid sequence, a new amino acid sequence composed of 21 amino acids is added after the alanine (Ala) at position 112 of the amino acid sequence of OCIF (Sequence No. 5 in the Sequence Table).

OCIF5

The cytidine at position 9 of the base sequence of OCIFcDNA (Sequence No. 6) is converted to guanine, and in the amino acid sequence, the asparagine (Asn) at position -19 of the amino acid sequence of OCIF (Sequence No. 5 in the Sequence Table) is changed into lysine (Lys). However, this is an amino acid conversion in the signal sequence, and it is not thought to have any effect on secreted OCIF5.

The posterior half of an intron 2 of approximately 1.8 kb is inserted between positions 400 and 401 of the base sequence of OCIFcDNA (Sequence No. 6), and an open reading frame ends in this intron. In the amino acid sequence, a new amino acid sequence composed of 12 amino acids is added after the alanine (Ala) of position 112 of the amino acid sequence of OCIF (Sequence No. 5 in the Sequence Table).

[Practical Example 21]

Production of OCIF analogs (variants)

i) Production of expression plasmids of OCIF variant cDNA

Among the OCIF variant cDNA obtained in Practical Example 20, the plasmids pBKOCIF2 and pBKOCIF3, containing inserted cDNA of OCIF2 and 3 respectively, were digested with the restriction enzymes XhoI and BamHI (Takara Shuzo), the cDNA of OCIF2 and 3 respectively was cut out, isolation was carried out by agarose gel electrophoresis, and purification was carried out using a QIAEX Gel Extraction Kit (Qiagen Co.). The cDNA of this OCIF2 and 3 was inserted into the expression plasmid pCEP4 (Invitrogen Co.) which had been digested in advance using the restriction enzymes XhoI and BamHI (Takara Shuzo) using the Ligation Kit Ver. 2 (Takara Shuzo), and transformation of E. coli DH5 α (Gibco BRL Co.) was carried out.

Moreover, among the OCIF variant cDNA obtained in Practical Example 20, the plasmid pBKOCIF4, containing the inserted cDNA of OCIF4, was digested with the restriction enzymes SpeI and XhoI (Takara Shuzo), and after isolation by agarose gel electrophoresis, purification was carried out using a QIAEX Gel Extraction Kit (Qiagen Co.). This cDNA of OCIF4 was inserted into the expression plasmid pCEP4 (Invitrogen Co.), which had been digested in advance with the restriction enzymes NagI and XhoI (Takara Shuzo) using the Ligation Kit Ver. 2 (Takara Shuzo), and transformation of *E. coli* DH5 α (Gibco BRL Co.) was carried out.

Moreover, among the OCIF variant cDNA obtained in Practical Example 20, the plasmid pBKOCIF5, containing the inserted cDNA of OCIF5, was digested with the restriction enzyme Hind III (Takara Shuzo), the 5' region of the coding region of OCIF5 cDNA was cut out, and after isolation by agarose gel electrophoresis, purification was carried out using a QIAEX Gel Extraction Kit (Qiagen Co.). The OCIF expression plasmid pCEPOCIF obtained in Practical Example 13-i) was digested with the restriction enzyme Hind III (Takara Shuzo), the 5' region of the coding region of OCIFcDNA was removed, the DNA fragment pCEPOCIF-3', containing the pCEP plasmid and the 3' region of OCIFcDNA, was isolated by agarose gel electrophoresis, and purification was then carried out using the QIAEX Gel Extraction Kit (Qiagen Co.). This Hind III fragment of the OCIF5 cDNA was inserted into pCEPOCIF-3' using the Ligation Kit Ver. 2 (Takara Shuzo), and transformation of *E. coli* DH5 α (Gibco BRL Co.) was carried out.

The transformed strain obtained was caused to proliferate, and the expression plasmids pCEPOCIF 2, 3, 4, and 5, containing the inserted cDNA of OCIF2, 3, 4, and 5 respectively, were purified using a Qiagen column (Qiagen Co.). After the OCIF variant expression plasmid was caused to precipitate using ethanol, it was dissolved in sterile distilled water, and the following procedure was followed.

ii) Transient expression of OCIF variant cDNA and assaying of its activity

Using the OCIF variant expression plasmids pCEPOCIF 2, 3, 4, and 5 obtained in Practical Example 21-i), OCIF variants were transiently expressed according to the method described under Practical Example 13-ii), and their activity was investigated. The results showed that these OCIF variants were found to have weak activity.

[Practical Example 22]

Preparation of OCIF mutants

i) Preparation of plasmid vectors for OCIF mutant cDNA subcloning use

5 μ g of the plasmid vector mentioned under Practical Example 11 was cut with the restriction enzymes BamHI and XhoI (Takara Shuzo). The cut DNA was subjected to agarose gel electrophoresis for preparation use. A DNA fragment of approximately 1.6 kilobase pairs (kb)

containing the entire length of the OCIFcDNA was isolated, purification was carried out using the QIAEX Gel Extraction Kit (Qiagen Co.), and this was dissolved in 20 µl of sterilized distilled water to obtain DNA solution 1. Next, 3 µg of pBluescript IISK⁺ (Stratagene Co.) was cut with the restriction enzymes BamHI and XhoI (Takara Shuzo). The cut DNA was then subjected to agarose gel electrophoresis for preparation use. A DNA fragment of approximately 3.0 kb was isolated, purification was carried out using the QIAEX Gel Extraction Kit (Qiagen Co.), and this was dissolved in 20 µl of sterilized distilled water to obtain DNA solution 2. 1 µl of DNA solution 2 and 4 µl of DNA solution 1 were mixed, 5 µl of DNA Ligation Kit Ver. 2 I solution (Takara Shuzo) was added to this to make a mixed solution, the solution was heated for 30 minutes at 16°C, and a ligation reaction was carried out. Moreover, all of the ligation reactions described below were also carried out for 30 minutes with the temperature maintained at 16°.

Using this ligation reaction solution, transformation of *E. coli* was carried out under the following conditions. Moreover, all subsequent transformation of *E. coli* was carried out under the following conditions. 5 µl of this ligation reaction solution and 100 µl of *E. coli* DH5α competent cells (Gibco BRL Co.) were mixed in a 15 ml sterilization tube (Iwashiro Glass Co.), and the mixture was then left in ice water for 30 minutes. After heating for 45 seconds at 42°C, 250 µl of L culture medium (1% tryptophan, 0.5% yeast extract, 1% NaCl) was added, and culturing was then carried out while stirring at 37°C. 50 µl of bacterial solution was spread onto 2 ml of L agar culture medium containing 50 µg/ml of ampicillin. Culturing was carried out overnight at 37°C, and the 6 types of colonies which grew were then further cultured overnight in 2 ml of L ampicillin liquid culture medium, and the structure of the plasmids containing the various strains was investigated. A plasmid was obtained having a structure composed of a DNA fragment of approximately 1.6 kb containing the entire length of OCIFcDNA inserted at the BamHI XhoI cutting site of pBluescript IISK⁺ (referred to in the following as pSK⁺-OCIF).

ii) Preparation of mutant with Cys converted to Ser

(1) Introduction of mutation

Mutants were prepared in which, in the amino acid sequence shown in Sequence No. 4 of the Sequence Table, the Cys residues at positions 174, 181, 256, 298, and 379 were converted to Ser residues. The mutant in which the 174 Cys was converted to Ser was designated OCIF-C19S, that in which 181 Cys was converted to Ser was designated OCIF-C20S, that in which 256 Cys was converted to Ser was designated OCIF-C21S, that in which 298 Cys was converted to Ser was designated OCIF-C22S, and that in which 379 Cys was converted to Ser was designated OCIF-C23S2. In order to prepare these mutants, the base sequences coding for the various Cys residues were first converted into base sequences coding for Ser residues. Introduction of mutations

was carried out by means of a two-stage PCR (polymerase chain reaction). In the following, this will be referred to as a two-stage PCR reaction. The first stage consists of 2 PCR reactions (PCR 1 and PCR 2).

PCR 1 reaction solution

10X Ex Taq buffer (Takara Shuzo)	10 μ l
2.5 mM dNTP solution	8 μ l
Plasmid vector of Practical Example 11 (8 ng/ml)	2 μ l
Sterilized distilled water	73.5 μ l
20 μ M primer-1	5 μ l
100 μ M primer-2 (for introduction of mutation)	1 μ l
Ex Taq (Takara Shuzo)	0.5 μ l

PCR 2 reaction solution

10X Ex Taq buffer (Takara Shuzo)	10 μ l
2.5 mM dNTP solution	8 μ l
Plasmid vector of Practical Example 11 (8 ng/ml)	2 μ l
Sterilized distilled water	73.5 μ l
20 μ M primer-3	5 μ l
100 μ M primer-4 (for introduction of mutation)	1 μ l
Ex Taq (Takara Shuzo)	0.5 μ l

In introducing the various mutations, only the type of primer was changed, and the other components of the reaction were left the same. The primers used in the various reactions are shown in Table 10 and their sequences are shown in Sequence Nos. 20, 23, 27, and 30-40 of the Sequence Table. After PCR 1 reaction solution and PCR 2 reaction solution had been placed in separate microcentrifugation tubes and mixed, PCR was carried out under the following conditions. After treatment for 3 minutes at 97°C, the reaction was repeated 25 times in the 3 stages of 1 minute at 95°C, 1 minute at 55°C, and 3 minutes at 72°C, and the temperature was then maintained at 70°C for 5 minutes. A portion of the reaction solution was subjected to agarose gel electrophoresis, and it was confirmed that a DNA fragment of the target length had been synthesized. After completion of the first-stage PCR reaction, the primer was removed from the reaction solution using the Amicon Microcon (Amicon Co.), the final solution volume was adjusted to 50 μ l using sterilized distilled water, and the stage-two PCR reaction (PCR 3) was then carried out using the

DNA fragment obtained.

PCR 3 reaction solution

10X Ex Taq buffer (Takara Shuzo)	10 µl
2.5 mM dNTP solution	8 µl
DNA fragment obtained by PCR 1	5 µl
DNA fragment obtained by PCR 2	5 µl
Sterilized distilled water	61.5 µl
20 µM primer-1	5 µl
20 µM primer-3	5 µl
Ex Taq (Takara Shuzo)	0.5 µl

Table 10

Mutant name	Primer-1	Primer-2	Primer-3	Primer-4
OCIF-C19S	IF 10	C19SR	IF 3	C19SF
OCIF-C20S	IF 10	C20SR	IF 3	C20SF
OCIF-C21S	IF 10	C21SR	IF 3	C21SF
OCIF-C22S	IF 10	C22SR	IF 14	C22SF
OCIF-C23S	IF 6	C23SR	IF 14	C23SF

After the above solution was placed in a microcentrifugation tube and mixed, PCR was conducted under the same conditions as for PCR 1 and PCR 2. A portion of the reaction solution was subjected to agarose (1% or 1.5%) electrophoresis, and it was confirmed that a DNA fragment of the target length had been synthesized. The DNA obtained by PCR was caused to precipitate using ethanol, dried in a vacuum, and dissolved in 40 µl of sterilized distilled water. The solution containing the C19S mutant DNA fragment was designated solution A, that containing the C20S mutant DNA fragment was designated solution B, that containing the C21S mutant DNA fragment was designated solution C, that containing the C22S mutant DNA fragment was designated solution D, and that containing the C23S mutant DNA fragment was designated solution E.

A DNA fragment in 20 µl of solution A was cut using the restriction enzymes NdeI and SphI (Takara Shuzo). A DNA fragment of approximately 400 bp was isolated and purified by means of electrophoresis for preparation use and then dissolved in 20 µl of distilled water (DNA solution 3). Next, 2 µg of pSK⁺-OCIF was cut with the restriction enzymes NdeI and SphI (Takara Shuzo), and a DNA fragment of approximately 4.2 kb was isolated and purified by electrophoresis for

preparation use and then dissolved in 20 μ l of sterilized distilled water (DNA solution 4). 2 μ l of DNA solution 3 and 3 μ l of DNA solution 4 were mixed, 5 μ l of DNA Ligation Kit Ver. 2 I was added, and a ligation reaction was carried out. After the reaction, using 5 μ l of the ligation solution, transformation of *E. coli* DH5 α was carried out. A strain having the target plasmid DNA was then selected based on analysis of DNA structure from the ampicillin-resistant converted cells obtained. DNA structure was analyzed by means of measuring the length of the fragment obtained through restriction enzyme cutting and by determining its base sequence. The target plasmid DNA obtained was designated pSK-OCIF-C19S.

The C20S mutant DNA fragment in 20 μ l of solution B was cut with the restriction enzyme NdeI and SphI (Takara Shuzo). A DNA fragment of approximately 400 bp was isolated and purified by electrophoresis for preparation use and then dissolved in 20 μ l of distilled water (DNA solution 5). 2 μ l of DNA solution 5 and 3 μ l of DNA solution 4 were mixed, 5 μ l of DNA Ligation Kit Ver. 2 I solution was added, and a ligation reaction was carried out. After the reaction, using 5 μ l of the ligation solution, conversion of *E. coli* DH5 α was carried out. A strain having the target plasmid DNA was selected by DNA structural analysis from the ampicillin-resistant converted cells obtained. DNA analysis was carried out by means of measuring the length of the fragment obtained by restriction enzyme cutting and by determining its base sequence. The target plasmid DNA obtained was designated pSK-OCIF-C20S.

A DNA fragment in 20 μ l of solution C was cut using the restriction enzymes NdeI and SphI (Takara Shuzo). A DNA fragment of approximately 400 bp was isolated and purified by electrophoresis for preparation use and then dissolved in 20 μ l of distilled water (DNA solution 6). 2 μ g of DNA solution 6 and 3 μ l of DNA solution 4 were mixed, 5 μ l of DNA Ligation Kit Ver. 2 I was added, and a ligation reaction was carried out. After the reaction, using 5 μ l of the ligation solution, conversion of *E. coli* DH5 α was carried out. A strain having the target plasmid DNA was selected by means of DNA structural analysis from the ampicillin-resistant transformed cells obtained. The DNA structure was analyzed by means of measuring the length of the fragment obtained by restriction enzyme cutting and determining its base sequence. The target plasmid DNA obtained was designated pSK-OCIF-C21S.

A DNA fragment in 20 μ l of solution D was cut with restriction enzymes NdeI and BstPI (Takara Shuzo). A DNA fragment of approximately 600 bp was isolated and purified by means of electrophoresis for preparation use and dissolved in 20 μ l of distilled water (DNA solution 7). Next, 2 μ g of pSK⁺-OCIF was cut with the restriction enzymes NdeI and BstPI (Takara Shuzo), and a DNA fragment of approximately 4.0 kb was isolated and purified by electrophoresis for preparation use and then dissolved in 20 μ l of distilled water (DNA solution 8). 2 μ l of DNA

solution 7 and 3 μ l of DNA solution 8 were mixed, 5 μ l of DNA Ligation Kit Ver. 2 I was added, and a ligation reaction was carried out. After the reaction, using 5 μ l of the ligation solution, transformation of *E. coli* DH5 α was carried out. A strain having the target plasmid DNA was selected by means of DNA structural analysis from the ampicillin-resistant transformed cells obtained. Analysis of DNA structure was carried out by measuring the length of the fragment obtained by restriction enzyme cutting and determining its base sequence. The target plasmid DNA obtained was designated pSK-OCIF-C22S.

A DNA fragment in 20 μ l of solution E was cut using the restriction enzymes BstPI and EcoRV (Takara Shuzo). A DNA fragment of approximately 120 bp was isolated and purified by electrophoresis for preparation use and then dissolved in 20 μ l of sterilized distilled water (DNA solution 9). Next, 2 μ g of pSK⁺-OCIF was cut using the restriction enzymes BstEII and EcoRV (Takara Shuzo), and a DNA fragment of approximately 4.5 kb was isolated and purified by electrophoresis for preparation use and then dissolved in 20 μ l of distilled water (DNA solution 10). 2 μ l of DNA solution 9 and 3 μ l of DNA solution 10 were mixed, 5 μ l of DNA Ligation Kit Ver. 2 I was added, and a ligation reaction was carried out. After the reaction, using 5 μ l of the ligation solution, transformation of *E. coli* DH5 α was carried out. A strain having the target plasmid DNA was selected by means of DNA structural analysis from the ampicillin-resistant transformed cells obtained. Analysis of DNA structure was carried out by measuring the length of the fragment obtained by restriction enzyme cutting and determining its base sequence. The target plasmid DNA obtained was designated pSK-OCIF-C23S.

(2) Construction of mutant expression vectors

The target plasmid DNA obtained (pSK-OCIF-C19S, pSK-OCIF-C20S, pSK-OCIF-C21S, pSK-OCIF-C22S, and pSK-OCIF-C23S) was cut using the restriction enzymes BamHI and XhoI (Takara Shuzo), a DNA fragment measuring approximately 1.6 kb containing the entire length of OCIFcDNA (containing the target mutation) was isolated and purified, and then dissolved in 20 μ l of sterilized distilled water. These solutions were designated C19SDNA, C20SDNA, C21SDNA, C22SDNA, and C23SDNA respectively. Next, 5 μ g of the expression vector pCEP4 (Invitrogen Co.) was cut with the restriction enzymes BamHI and XhoI (Takara Shuzo), and DNA of approximately 10 kb was isolated and purified and then dissolved in 40 μ l of sterilized distilled water (pCEP4DNA solution). 1 μ l of pCEP4DNA solution and 6 μ l each of C19SDNA solution, C20SDNA solution, C21SDNA solution, C22SDNA solution, and C23SDNA solution were separately mixed, 7 μ l of DNA Ligation Kit Ver. 2 I solution was added to the various mixed solutions, and ligation reactions were carried out. After completion of the reactions, using 7 μ l of reaction solution, 100 μ l of *E. coli* DH5 α competent cell solution was transformed. A total of 5

strains having plasmid DNA of the target structure, with various DNA fragments of approximately 1.6 kb inserted at the XhoI and BamHI sites of pCEP4 was selected from the ampicillin-resistant transformed cells obtained, and these were designated pCEP4-OCIF-C19S, pCEP4-OCIF-C20S, pCEP4-OCIF-C21S, pCEP4-OCIF-C22S, and pCEP4-OCIF-C23S.

ii) Preparation of domain deletion mutant

(1) Introduction of domain deletion mutation

Of the amino acids shown in Sequence No. 4, mutants were prepared having the sections from Thr at position 2 to Ala at position 42, Pro at position 43 to Cys at position 84, Glu at position 85 to Lys at position 122, Arg at position 123 to Cys at position 164, Asp at position 177 to Gln at position 251, and Ile at position 253 to His at position 326 respectively deleted. The mutant with the sequence from Thr at position 2 to Ala at position 42 deleted was designated OCIF-DCR1, that with the sequence from Pro at position 43 to Cys at position 84 deleted was designated OCIF-DCR2, that with the sequence from Glu at position 85 to Lys at position 122 deleted was designated OCIF-DCR3, that with the sequence from Arg at position 123 to Cys at position 164 deleted was designated OCIF-DCR4, that with the sequence from Asp at position 177 to Gln at position 251 deleted was designated OCIF-DDD1, and that with the sequence from Ile at position 253 to His at position 326 deleted was designated OCIF-DDD2. The introduction of domain deletion mutants was also carried out by the two-stage PCR method as described under Practical Example 22-ii). The primers used in the various mutation introduction reactions are shown in Table 11, and their sequences are shown in Sequence Nos. 19, 25, 40-53, and 54 of the Sequence Table.

Table 11

Mutant name	Primer-1	Primer-2	Primer-3	Primer-4
OCIF-DCR1	XhoI F	DCR1R	IF 2	DCR1F
OCIF-DCR2	XhoI F	DCR2R	IF 2	DCR2F
OCIF-DCR3	XhoI F	DCR3R	IF 2	DCR3F
OCIF-DCR4	XhoI F	DCR4R	IF 16	DCR4F
OCIF-DDD1	IF 8	DDD1R	IF 14	DDD1F
OCIF-DDD2	IF 8	DDD2R	IF 14	DDD2F

The DNA obtained by PCR was caused to precipitate with ethanol, dried in a vacuum, and dissolved in 40 µl of sterilized distilled water. Solution containing the DCR1 mutant DNA fragment was designated solution F, that containing the DCR2 mutant DNA fragment was designated solution G, that containing the DCR3 mutant DNA fragment was designated solution H, that containing the

DCR4 mutant DNA fragment was designated solution I, that containing the DDD1 mutant DNA fragment was designated solution J, and that containing the DDD2 mutant DNA fragment was designated solution K.

A DNA fragment in 20 μ l of solution F was cut using the restriction enzymes NdeI and XhoI (Takara Shuzo). A DNA fragment of approximately 500 bp was isolated and purified by electrophoresis for purification use and dissolved in 20 μ l of sterilized distilled water (DNA solution 11). Next, 2 μ g of pSK⁺-OCIF was cut with the restriction enzymes NdeI and XhoI (Takara Shuzo), and a DNA fragment of approximately 4.0 kb was isolated and purified by electrophoresis for preparation use and dissolved in 20 μ l of sterilized distilled water (DNA solution 12). 2 μ l of DNA solution 11 and 3 μ l of DNA solution 12 were mixed, and 5 μ l of DNA Ligation Kit Ver. 2 I was added, and a ligation reaction was carried out. After the reaction, using 5 μ l of the ligation solution, transformation of *E. coli* DH5 α was carried out. A strain having plasmid DNA with the target mutant introduced into OCIFcDNA was selected by means of DNA structural analysis from the ampicillin-resistant transformed cells obtained. Structural analysis of DNA was carried out by measuring the length of the fragment obtained by restriction enzyme cutting and determining its base sequence. The target plasmid DNA obtained was designated pSK-OCIF-DCR1. A DNA fragment in 20 μ l of solution G was cut with the restriction enzymes NdeI and XhoI (Takara Shuzo). A DNA fragment of approximately 500 bp was isolated and purified by electrophoresis for preparation use and then dissolved in 20 μ l of sterilized distilled water (DNA solution 13). 2 μ l of DNA solution 13 and 3 μ l of DNA solution 12 were mixed, 5 μ l of DNA Ligation Kit Ver. 2 I was added, and a ligation reaction was carried out. After the reaction, using 5 μ l of the ligation solution, transformation of *E. coli* DH5 α was carried out. A strain having the target plasmid was selected by DNA structural analysis from the ampicillin-resistant transformed cells obtained. Structural analysis of DNA was carried out by measuring the length of the fragments obtained by restriction enzyme cutting and determining their base sequence. The target plasmid DNA obtained was designated pSK-OCIF-DCR2.

A DNA fragment in 20 μ l of solution H was cut using the restriction enzymes NdeI and XhoI (Takara Shuzo). A DNA fragment of approximately 500 bp was isolated and purified by means of electrophoresis for preparation use and then dissolved in 20 μ l of sterilized distilled water (DNA solution 14). 2 μ l of DNA solution 14 and 3 μ l of DNA solution 12 were mixed, 5 μ l of DNA Ligation Kit Ver. 2 I was added, and a ligation reaction was carried out. After the reaction, using 5 μ l of the ligation solution, transformation of *E. coli* DH5 α was carried out. A strain having plasmid DNA with the target mutant introduced into OCIFcDNA was selected by means of DNA structural analysis from the ampicillin-resistant transformed cells obtained. Structural analysis of DNA was

carried out by measuring the length of the fragments obtained by restriction enzyme cutting and determining their base sequence. The target plasmid DNA obtained was designated pSK-OCIF-DCR3.

A DNA fragment in 20 μ l of solution I was cut using the restriction enzymes XhoI and SphI (Takara Shuzo). A DNA fragment of approximately 900 bp was isolated and purified by electrophoresis for preparation use and then dissolved in 20 μ l of sterilized distilled water (DNA solution 15). Next, 2 μ l of pSK⁺-OCIF was cut using the restriction enzymes XhoI and SphI (Takara Shuzo), and a DNA fragment of approximately 3.6 kb was isolated and purified by means of electrophoresis for preparation use and then dissolved in 20 μ l of sterilized distilled water (DNA solution 16). 2 μ l of DNA solution 15 and 3 μ l of DNA solution 16 were mixed, 5 μ l of DNA Ligation Kit Ver. 2 I was added, and a ligation reaction was carried out. After the reaction, using 5 μ l of the ligation solution, transformation of *E. coli* DH5 α was carried out. A strain having the target plasmid DNA was selected by DNA structural analysis from the ampicillin-resistant transformed cells obtained. Structural analysis of the DNA was carried out by measuring the length of the fragment obtained by restriction enzyme cutting and determining its base sequence. The target plasmid DNA obtained was designated pSK-OCIF-DCR4.

A DNA fragment in 20 μ l of solution J was cut using the restriction enzymes BstPI and NdeI (Takara Shuzo). A DNA fragment of approximately 400 bp was isolated and purified by electrophoresis for preparation use and then dissolved in 20 μ l of sterilized distilled water (DNA solution 17). 2 μ l of DNA solution 17 and 3 μ l of DNA solution 8 were mixed, 5 μ l of DNA Ligation Kit Ver. 2 I was added, and a ligation reaction was carried out. After the reaction, using 5 μ l of the ligation solution, transformation of *E. coli* DH5 α was carried out. A strain of the target plasmid DNA was selected by DNA structural analysis from the ampicillin-resistant transformed cells obtained. DNA structural analysis was carried out by measuring the length of the fragments obtained by restriction enzyme cutting and determining their base sequence. The target plasmid DNA obtained was designated pSK-OCIF-DDD1.

A DNA fragment in 20 μ l of solution K was cut using the restriction enzymes BstPI and NdeI (Takara Shuzo). A DNA fragment of approximately 400 bp was isolated and purified by electrophoresis for preparation use and then dissolved in 20 μ l of sterilized distilled water (DNA solution 18). 2 μ l of DNA solution 18 and 3 μ l of DNA solution 8 were mixed, 5 μ l of DNA Ligation Kit Ver. 2 I was added, and a ligation reaction was carried out. After the reaction, using 5 μ l of the ligation solution, transformation of *E. coli* DH5 α was carried out. A strain having the target plasmid DNA was selected by DNA structural analysis from the ampicillin-resistant transformed cells obtained. Structural analysis of the DNA was carried out by measuring the length

of the fragments obtained by restriction enzyme cutting and determining their base sequence. The target plasmid DNA obtained was designated pSK-OCIF-DDD2.

(2) Construction of mutant expression vectors

The target plasmid DNA obtained (pSK-OCIF-DCR1, pSK-OCIF-DCR2, pSK-OCIF-XR3, pSK-OCIF-DCR4, pSK-OCIF-DDD1, and pSK-OCIF-DDD2) was cut using the restriction enzymes BamHI and XhoI (Takara Shuzo), DNA fragments of approximately 1.4-1.5 kb containing the entire length of OCIFcDNA (including the target mutation) were isolated and purified, and they were then dissolved in 20 µl of sterilized distilled water. These were designated DCR1DNA solution, DCR2DNA solution, DCR3DNA solution, DCR4DNA solution, DDD1DNA solution, and DDD2DNA solution. 1 µl of the pCEP4DNA solution of Practical Example 22-ii) and 6 µl each of DCR1DNA solution, DCR2DNA solution, DCR3DNA solution, DCR4DNA solution, DDD1DNA solution, and DDD2DNA solution were separately mixed, 7 µl of DNA ligation buffer was added to the various mixed solutions, and a ligation reaction was carried out. After completion of the reaction, using 7 µl of reaction solution, transformation of *E. coli* DH5α was carried out. A total of 6 strains of plasmid DNA having structures in which fragments of 1.4-1.5 kb are inserted at pCEP4 BamHI and XhoI sites were selected from the ampicillin-resistant transformed cells obtained. The plasmids having the target structure were designated pCEP4-OCIF-DCR1, pCEP4-OCIF-DCR2, pCEP4-OCIF-DCR3, pCEP4-OCIF-DCR4, pCEP4-OCIF-DDD1, and pCEP4-OCIF-DDD2 respectively.

iii) Preparation of C terminal domain deletion mutant

(1) Introduction of C terminal domain deletion mutation

Of the amino acids shown in Sequence No. 4, mutants were prepared having the sequences from the Cys at position 379 to Leu at position 380, the Ser at position 331 to the Leu at position 380, the Asp at position 252 to the Leu at position 380, the Asp at position 177 to the Leu at position 380, the Arg at position 123 to the Leu at position 380, and the Cys at position 86 to the Leu at position 380 deleted. The mutant having the sequence from Cys at position 379 to Leu at position 380 deleted was designated OCIF-CL, that with the sequence from Ser at position 331 to Leu at position 380 deleted was designated OCIF-CC, that with the sequence from Asp at position 252 to Leu at position 380 deleted was designated OCIF-CDD2, that with the sequence from Asp at position 177 to Leu at position 380 deleted was designated OCIF-CDD1, that with the sequence from Arg at position 123 to Leu at position 380 deleted was designated OCIF-CCR4, and that with the sequence from Cys at position 86 to Leu at position 380 deleted was designated OCIF-CCR3 respectively.

Introduction of mutation for the preparation of mutant OCIF-CL was carried out by means

of the two-stage PCR method described under Practical Example 22-ii). The primers used in the mutation introduction reaction are shown in Table 12, and their base sequences are shown in Sequence Nos. 23, 40, 55, and 56 of the Sequence Table. The DNA obtained by PCR was caused to precipitate with ethanol, dried in a vacuum, and then dissolved in 40 μ l of sterilized distilled water (solution L).

A DNA fragment in 20 μ l of solution L was cut using the restriction enzymes BstPI and EcoRV (Takara Shuzo). A DNA fragment of approximately 100 bp was isolated and purified by electrophoresis and then dissolved in 20 μ l of sterilized distilled water (DNA solution 19). Next, 2 μ l of DNA solution 9 and 3 μ l of the DNA solution 10 described under Practical Example 22-ii) were mixed, 5 μ l of DNA Ligation Kit Ver. 2 I was added, and a ligation reaction was carried out. After the reaction, using 5 μ l of the ligation solution, transformation of E. coli DH5 α was carried out. A strain having the target plasmid DNA was selected by DNA structural analysis from the ampicillin-resistant transformed cells obtained. Structural analysis of DNA was carried out by measuring the length of the fragment obtained by restriction enzyme cutting and determining its base sequence. The target plasmid DNA obtained was designated pSK-OCIF-CL. In introduction of mutation for the preparation of the mutant OCIF-CC, OCIF-CDD2, OCIF-CDD1, OCIF-CCR4, and OCIF-CCR3, the one-stage PCR method was used. The reaction conditions were as follows.

PCR reaction solution for introduction of C terminal domain deletion mutation

10X Ex Taq buffer (Takara Shuzo)	10 μ l
2.5 mM dNTP solution	8 μ l
Plasmid vector of Practical Example 11 (8 ng/ml)	2 μ l
Sterilized distilled water	73.5 μ l
20 μ M primer OCIF Xho F	5 μ l
100 μ M primer for introduction of mutation	1 μ l
Ex Taq (Takara Shuzo)	0.5 μ l

Table 12

Mutant name	Primer-1	Primer-2	Primer-3	Primer-4
OCIF-CL	IF6	CL R	IF 14	CL F

In introduction of the various mutations, only the type of primer was changed, and the other reaction components were left the same. The primers used for introduction of mutation in the various reactions are shown in Table 13, and their sequences are shown in Sequence Nos. 57-61 in the Sequence Table. After the PCR reaction solution was placed in a microcentrifugation

tube and mixed, PCR was conducted under the following conditions. After treatment for 3 minutes at 97°C, the reaction was repeated 25 times in the 3 stages of 30 seconds at 95°C, 30 seconds at 50°C, and 3 minutes at 70°C, afterwards the temperature was maintained at 70°C for 5 minutes. A portion of the reaction solution was subjected to agarose gel electrophoresis, and it was confirmed that a DNA fragment of the target length had been synthesized. The primer was removed from the reaction solution using an Amicon Microcon (Amicon Co.), the DNA was caused to precipitate using ethanol, dried in a vacuum, then dissolved in 40 µl of sterilized distilled water. DNA fragments in 20 µl of solution containing the various mutant DNA fragments were cut by means of the restriction enzymes XhoI and BamHI. After completion of enzyme cutting, the DNA was caused to precipitate with ethanol, dried in a vacuum, and dissolved in 20 µl of sterilized distilled water. The solutions were designated CC DNA solution, CDD2 DNA solution, CDD1 DNA solution, CCR4 DNA solution, and CCR3 DNA solution respectively.

Table 13

Mutant name	Primer used for introduction of mutation
OCIF-CC	CC R
OCIF-CDD2	CDD2 R
OCIF-CDD1	CDD1 R
OCIF-CCR4	CCR4 R
OCIF-CCR3	CCR3 R

(2) Construction of mutant expression vectors

pSK-OCIF-CL was cut using the restriction enzymes BamHI and XhoI (Takara Shuzo). a DNA fragment of approximately 1.5 kb containing OCIFcDNA (including the target mutation) was isolated and purified, and it was then dissolved in 20 µl of sterilized distilled water (CLDNA solution). 1 µl of the pCEP4 DNA solution described under Practical Example 22-ii) was separately mixed with 6 µl each of CLDNA solution, CCDNA solution, CDD2DNA solution, CDD1DNA solution, CCR4DNA solution, and CCR3DNA solution, 7 µl of DNA Ligation Kit Ver. 2 I solution was added, and a ligation reaction was carried out. After completion of the reaction, using 7 µl of the reaction solution, transformation of E. coli DH5α was carried out. A total of 6 strains of plasmid DNA having a structure in which OCIFcDNA fragments having the target mutation were inserted at the XhoI-BamHI site of pCEP4 were selected from the ampicillin-resistant transformed cells obtained. The plasmids having the target structure were designated pCEP4-OCIF-CL, pCEP4-OCIF-CC, pCEP4-OCIF-CDD2, pCEP4-OCIF-CDD1, pCEP4-OCIF-CCR4, and

pCEP4-OCIF-CCR3 respectively.

(v) Preparation of C terminal deletion mutant

(1) Introduction of C terminal deletion mutation

In the amino acids shown in Sequence No. 4, a mutant with the sequence from Gln at position 371 to Leu at position 380 deleted, and the 2 residues of Leu-Val added (OCIF-CBst), a mutant with the sequence from Cys at position 298 to Leu at position 30 deleted and the residues of Ser-Leu-Asp added (OCIF-CSph), a mutant with the sequence from Asn at position 167 to Leu at position 380 deleted (OCIF-CBsp), and a mutant with the sequence from Cys at position 62 to Leu at position 30 deleted and the two residues Leu-Val added (OCIF-CPst) were prepared. 2 µg each of pSK⁺-OCIF was cut using the restriction enzymes BstPI, SphI, PstI (Takara Shuzo), and BspEI (New England Biolabo Co.), the DNA was purified by means of phenol treatment and ethanol precipitation, and it was then dissolved in 10 µl of sterilized distilled water. Using 2 µl portions of the solution, the terminals of the various DNA was smoothed using a DNA Branding Kit (Takara Shuzo) (final volume 5 µl). 1 µg (1 µl) of an XbaI linker containing amber cotton (5'-CTAGTCTAGACTAG-3') and 6 µl of DNA Ligation Kit Ver. 2 I solution were added to this reaction solution and a ligation reaction was carried out. After the reaction, using 6 µl of the ligation solution, transformation of E. coli DH5α was carried out. A strain of the target plasmid DNA was selected by DNA structural analysis from the ampicillin-resistant transformed bacteria obtained. Structural analysis of the DNA was carried out by measuring the length of the fragments obtained by restriction enzyme cutting and determining their base sequences. The types of target plasmid DNA obtained were designated pSK-OCIF-CBst, pSK-OCIF-CSph, pSK-OCIF-CBsp, and pSK-OCIF-CPst.

(2) Construction of mutant expression vectors

The plasmid DNA obtained (pSK-OCIF-CBst, pSK-OCIF-CSph, pSK-OCIF-CBsp, and pSK-OCIF-CPst) were cut with the restriction enzymes BamHI and XhoI (Takara Shuzo), a DNA fragment of approximately 1.5 kilobase pairs (kb) containing the entire length of OCIFcDNA (including the target mutation) was isolated and purified, and it was then dissolved in 20 µl of sterilized distilled water (the solutions were designated CBstDNA solution, CSphDNA solution, CBspDNA solution, and CPstDNA solution respectively). 1 µl of the pCEP4 DNA solution mentioned under Practical Example 22-ii) and 6 µl each of CBstDNA solution, CSphDNA solution, CBspDNA solution, and CPstDNA solution were separately mixed, 7 µl of DNA Ligation Kit Ver. 2 I solution was added to the various mixed solutions, and a ligation reaction was carried out. After completion of the reaction, using 7 µl of reaction solution, transformation of E. coli DH5α was carried out. A total of 5 strains having plasmid DNA of a structure in which an OCIFcDNA

fragment having the target mutation was inserted between the XhoI and BamHI sites of pCEP4 were selected from the ampicillin-resistant transformed cells obtained. The plasmids having the target structure were designated pCEP4-OCIF-CBst, pCEP4-OCIF-CSph, pCEP4-OCIF-CBsp, and pCEP4-OCIF-CBst respectively.

v) Preparation of mutant expression vectors

E. coli having mutant expression vectors (total of 21 types) was caused to propagate, and then the various mutant expression vectors were purified using a Qiagen column (Qiagen Co.). After the various expression vectors were caused to precipitate using ethanol, they were dissolved in sterilized distilled water, and the following procedure was followed.

vi) Transient expression of mutant cDNA and assaying of its activity

Using the various OCIF mutant expression plasmids purified under Practical Example 22-v), OCIF mutants were expressed according to the method of Practical Example 13. In the following, only the items which were modified are discussed. In DNA introduction, a 24-well plate was used. 2×10^5 293/EBNA cells were seeded into the various wells using IMDM culture solution containing 10% fetal bovine serum. The amounts of the mutant expression vector and lipofectamine used in DNA introduction were 1 μ g and 4 μ l respectively. Dilution was carried out with OPTI-MEM culture medium (Gibco BRL Co.) to make a final volume of 0.5 ml. A mixed solution of the mutant expression vector and lipofectamine was added to the cells and incubation was carried out in a CO₂ incubator for 24 hours at 37°C, the mixed solution was then removed, 0.5 ml of Ex-cell 301 culture medium (JSR Co.) was added, and additional culturing was carried out in a CO₂ incubator for 48 hours at 37°C. The culture medium was recovered, and this was taken as a sample for assaying of mutant activity. The base sequences of the various mutants obtained are shown in Sequence Nos. 83-103 of the Sequence Table, and the amino acid sequences inferred from these sequences are shown in Sequence Nos. 62-82 of the Sequence Table respectively. Assaying of OCIF activity was carried out according to Practical Example 13. Moreover, the antigen volume of OCIF was determined according to the EIA method described under Practical Example 24. Table 14 shows activity per antigen volume compared to unmodified OCIF.

Name of mutant	Table 14 Activity
Unmodified OCIF	++
OCIF-C19S	+
OCIF-C20S	±
OCIF-C21S	±

OCIF-C22S	+	
OCIF-C23S	++	monomer C379S
OCIF-DCR1	±	
OCIF-DCR2	±	
OCIF-DCR3	±	
OCIF-DCR4	±	
OCIF-DDD1	+	
OCIF-DDD2	±	
OCIF-CL (22-399)	++	monomer
OCIF-CC (22-399)	++	
OCIF-CDD2 (22-272)	++	
OCIF-CDD1 (22-197)	+	
OCIF-CCR4 (22-143)	±	
OCIF-CCR3 (22-106)	±	
OCIF-CBst	++	
OCIF-CSph	++	
OCIF-CBsp (22-167)	±	
OCIF-CPst	±	

(In the table, ++ indicates activity per antigen volume exceeding 50% of the activity of unmodified OCIF, + indicates 10%-50%, and ± indicates less than 10% or that antigen volume cannot be precisely determined)

vi) Western blot analysis

10 µl of the sample used in assaying activity was subjected to Western blot analysis. 10 µl of sample buffer for SDS-PAGE use (0.5 M Tris-HCl, 20% glycerol, 4% SDS, 20 µg/ml bromophenol blue (pH 6.8)) was added to 10 ml of the sample, the mixture was boiled for 3 minutes at 100°C, and it was then subjected to 10% SDS-polyacrylamide electrophoresis under non-reductive conditions. After completion of phoresis, protein was blotted on a PVDF film (ProBlott[®], Perkin-Elmer Co.) using a semi-dry blotting unit (Biorad Co.). After blotting, the film was heated for 2 hours at 37°C together with the horseradish peroxidase-labeled anti-OCIF antibody mentioned in Practical Example 24. After washing, protein binding to anti-OCIF antibodies was detected using an ECL system (Amersham Co.). For OCIF, bands were detected at approximately 120 kilodaltons (kD) and 60 kD. Moreover, for OCIF-C23S, OCIF-CL, and OCIF-CC, bands were detected almost

exclusively at 60 kD. Furthermore, for OCIF-CDD2 and OCIF-CDD1, bands were detected in the form of main bands at approximately 40-50 kD and 30-40 kD respectively. Based on the above results, for OCIF, it became clear that the Cys residue at position 379 in the amino acid sequence of Sequence No. 4 in the Sequence Table was involved in dimer formation, that it also had activity with respect to monomers, and that even when the residues from Asp at position 177 to Leu at position 380 were deleted, it retained its activity.

[Practical Example 23]

Isolation of human OCIF genome DNA

D Screening of human genome DNA library

A genome library prepared using human placental chromosomal DNA and a lambda FIX II vector was purchased from Stratagene Co., and this was screened using OCIFcDNA as a probe. Screening was basically carried out according to the protocol enclosed with the genome library, but the general methods for handling phages, E. coli, and DNA were taken from Molecular Cloning: A Laboratory Manual.

After determining the titers of the purchased genome DNA library, 1×10^4 pfu of phages were infected with E. coli XL1-Blue MRA and then seeded on 20 plates (9 x 13 cm) together with 9 ml per plate of top agarose. After the plates were incubated overnight at 37°C, a Hybond-N nylon film (Amersham Co.) was placed on the agar plate, and the phages were transcribed. The nylon film with the transcribed phages was placed for 1 minute on the filter paper moistened with 1.5 M NaCl/0.5 M NaOH solution, it was then treated for 1 minute each with 1 M tris-HCl (pH 7.5) and 1.5 M NaCl/0.05 M tris-HCl (pH 7.5) and neutralized, and it was finally transferred to a piece of filter paper moistened with 2 XSSC. After this, by irradiating the nylon film with UV irradiation of 1,200 microjoules using a Stratalinker (Stratagene Co.), the phage DNA was fixed on the film. Next, this nylon film was immersed in rapid hybridization buffer (Amersham Co.) and pre-hybridization was carried out. After 1 hour of pre-hybridization, ^{32}P -labeled OCIFcDNA was added, and hybridization was carried out overnight at 65°C. This cDNA probe was prepared by cutting the plasmid pBKOCIF having OCIFcDNA of 1.6 kb obtained in Practical Example 11 with the restriction enzymes BamHI and XhoI, isolating the OCIFcDNA by agarose gel electrophoresis, and labeling this OCIFcDNA with ^{32}P using a Megaprimer DNA labeling system (Amersham Co.). Labeling was carried out according to the protocol enclosed with the labeling system. In hybridization, one probe was used per 1 ml of hybridization buffer and in the amount of 5×10^5 cpm. After hybridization, the nylon film was washed for 5 minutes at room temperature with 2 XSSC, and it was then washed 4 times for 20 minutes each at 65°C with 0.5 XSSC/0.1% SDS. After the 4 washings were carried out, the nylon film was dried it was subjected to autoradiography at -80°C

using Fuji Film x-ray film, super HR-H, and a sensitization screen. As 6 signals were detected on the autoradiogram, top agarose was cut out of the positions on the agar plate corresponding to the respective signals, it was then immersed in 0.5 ml of SM buffer to which 1% chloroform had been added and left standing overnight to extract the phages. The respective phage extract solutions were diluted 1,000 times with SM buffer, 1 ml and 20 ml were removed therefrom and again infected with the aforementioned *E. coli*, and then seeded onto the agar plates together with top agarose according to the above-described method. After the phages were transcribed onto the nylon film, pre-hybridization, hybridization, washing, drying, and autoradiography were carried out according to the above method. This operation of phage purification was carried out for all 6 signals detected in initial autoradiography, and this operation was repeated until all of the phage plaques on the agar plates had undergone hybridization with the cDNA probe. The purified phage plaques were cut out, immersed in 0.5 ml of SM buffer containing 1% chloroform, and stored at 4°C. The 6 purified phages obtained in this manner were designated lambda 0IF3, lambda 0IF8, lambda 0IF9, lambda 0IF11, lambda 0IF12, and lambda 0IF17 respectively.

ii) Analysis of human OCIF genome DNA clones by restriction enzyme digestion and southern blot hybridization

The DNA of the 6 types of purified phages was purified by the plate lysis method as described in Molecular Cloning: A Laboratory Manual. These types of DNA were digested using restriction enzymes, and the fragments obtained were isolated by agarose gel electrophoresis. Moreover, after the fragments isolated using agarose gel were transcribed onto a nylon film according to the general method, southern blot hybridization was carried out using OCIFcDNA as a probe. The results of this analysis clearly showed that the 6 types of purified phages were different clones. Among the DNA fragments obtained by restriction enzyme digestion, concerning those hybridized with OCIFcDNA, after subcloning in plasmid vectors, the base sequences were analyzed by the following method.

iii) Subcloning in plasmid vectors of DNA fragments obtained by restriction enzyme digestion from genome DNA clones and determination of their base sequences

Lambda 0IF8 was digested by the restriction enzymes *EcoRI* and *NotI*, and the fragments produced were isolated using 0.7% agarose gel. An *EcoRI/NotI* fragment of 5.8 kb was extracted from the gel using a QIAEX II Gel Extraction Kit (Qiagen Co.) according to the enclosed protocol. This fragment was subjected to ligation using a pBluescript II SK+ vector (Stratagene Co.) cut in advance using *EcoRI* and *NotI* (Stratagene Co.) and ready-to-go T4 ligase (Pharmacia Co.) according to the enclosed protocol. After the recombinant plasmid obtained was introduced into competent DH5 α *E. coli* (Pharmacia Co.), it was seeded onto an agarose plate containing 50 μ g/ml of

ampicillin, and *E. coli* containing the plasmid was selected. The recombinant plasmid containing the EcoRI/NotI fragment of 5.8 kb prepared in the above manner was designated pBSG8-5.8. Next, pBSG8-5.8 was digested using the restriction enzyme HindIII, the DNA fragment of 0.9 kb produced was isolated by means of agarose gel, extraction was carried out according to the above method, it was inserted into a pBluescriptII SK- vector (Stratagene Co.) cut in advance with HindIII, and cloning was carried out according to the above method. The recombinant plasmid having this HindIII fragment of 0.9 kb was designated BS8H0.9. On the other hand, DNA of lambda 0IF11 was digested using EcoRI, and after the fragments of 6 kb, 3.6 kb, and 2.6 kb produced were isolated, they were inserted into a pBluescriptII SK+ vector by the same method as described above, and cloning was carried out. The recombinant plasmids having EcoRI fragments of 6 kb, 3.6 kb, and 2.6 kb prepared in this manner were designated pBSG11-6, pBSG11-3.6, and pBSG11-2.6. Moreover, the 3 fragments of 2.2 kb, 1.1 kb, and 1.05 kb produced by digesting pBSG11-6 using the restriction enzyme HindIII were isolated by agarose gel electrophoresis, and they were then inserted at the HindIII site of pBluescriptII SK- and cloning was carried out. These recombinant plasmids having HindIII fragments of 2.2 kb, 1.1 kb, and 1.05 kb were designated pBSGH2.2, pBS6H1.1, and pBS6H1.05 respectively. In analyzing the base sequence of the genome DNA, an ABI Dye Deoxy Terminator Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Co.) and a 373 DNA Sequencing System (Applied Biosciences Co.) were used. pBSG8-5.8, pBS8H0.9, pBSG11-6, pBSG11-3.6, pBSG11-2.6, pBSGH2.2, pBSGH1.1, and pBSGH1.05 were prepared according to the method specified in Molecular Cloning: A Laboratory Manual, and used as a template for determining base sequences. The base sequence of human OCIF genome DNA is shown in Sequence Nos. 104 and 105 of the Sequence Table. The sequence of bases interposed between exon 1 and exon 2 has not necessarily been completely determined, and it has been confirmed that a nucleotide of approximately 17 kb is interposed between the base sequences shown in Sequence Nos. 104 and 105 of the Sequence Table.

[Practical Example 24]

Determination of OCIF by EIA

i) Preparation of rabbit anti-OCIF antibodies

Three male Japanese white rabbits (body weights 2.5-3.0 kg, purchased from Kitayama Labesu Co.) were immunized by single subcutaneous administrations of 1 ml each of an emulsion composed of a mixture of 200 µg/ml of rOCIF and an equivalent amount of Freund's complete adjuvant (Difco Co.). Immunization was carried out at 1-week intervals for a total of 6 times, and whole blood samples were taken 10 days after the final immunization. Antibodies were purified from the separated serum as described below. Specifically, ammonium sulfate was added to anti-serum

diluted 2 times with PBS to a final concentration of 40 w/v %, and the mixture was left standing for 1 hour at 4°C and then centrifuged for 20 minutes at 8,000 rpm to cause precipitation. The sediment was dissolved in a small amount of PBS, and after it was dialyzed with respect to PBS at 4°C, it was run on a protein G-sepharose column (Pharmacia Co.). After washing with PBS, the adsorbed immunoglobulin G was eluted with 0.1 M glycine hydrochloride buffer solution (pH 3.0), and it was then immediately adjusted to a neutral pH using 1.5 M tris-hydrochloric acid buffer solution (pH 8.7). After the eluted protein fractions were dialyzed with respect to PBS, absorbance was measured at 280 nm, and the concentration was determined ($E^{1\%}_{1\text{cm}}$ 13.5). Horseradish peroxidase-labeled anti-OCIF antibodies were prepared using a maleimide-activated peroxidase kit (Pierce Co.). Specifically, 80 µg of N-succinimide-S-acetylthioacetate was added to 1 mg of purified antibody, and a reaction was then carried out for 30 minutes at room temperature. 5 mg of hydroxylamine was then added to this mixture to carry out deacetylation, and the modified antibody was then fractionated on a polyacrylamide desalination column. The protein fraction was mixed with 1 mg of maleimide-activated peroxidase and reacted at room temperature for 1 hour to obtain enzyme-labeled antibody.

ii) Determination of OCIF by sandwich EIA

100 µl of rabbit anti-OCIF antibody (2 µg/ml, 50 mM carbonate buffer solution (pH 9.6)) was added to the wells of a 96-well microtiter plate (MaxiSorp Immunoplate, Nunc Co.), and the plate was left standing overnight at 4°C to solidify the antibody. 25% Block Ace (Snow Brand Milk Products Co.) prepared with PBS was added to the wells in amounts of 300 µl each, the plate was left standing for 1 hour at 37°C to induce blocking, the samples (100 µl/well) were added, and a reaction was carried out for 2 hours at room temperature. After washing 3 times with PBS (PBST) containing 0.05% Tween 20, horseradish peroxidase-labeled anti-OCIF antibody diluted 10,000 times was added in amounts of 100 µl each and incubation was carried out for 2 hours at room temperature. After washing 3 times with PBST, 100 µl of enzyme substrate solution (TMB, ScyTek Co.) was added, coloration was carried out at room temperature, and the reaction was stopped. Absorbance at 450 nm was measured using a microplate reader (Immunoreader NJ2000, Nihon Intermed Co.), and the OCIF concentration of the samples was determined based on a calibration curve prepared using purified recombinant OCIF as a standard. The OCIF calibration curve is shown in Fig. 13.

[Practical Example 25]

Anti-OCIF monoclonal antibody

i) Preparation of human OCIF antibody-producing hybridoma

Human fibroblast IMR-90 cells were cultured, and OCIF was purified from this culture

solution according to the method described under Practical Example 11. The purified OCIF was dissolved in PBS to a concentration of 10 µg/100 µl, and this solution was used to immunize BALB/c mice by intraperitoneal administration at 2-week intervals. In the initial and second immunizations, a mixture containing an equivalent amount of Freund's complete adjuvant was administered. On the third day after the final immunization, the spleen was extracted, B lymphocytes were isolated, and cell fusion with the murine myeloma cells P3x63-AG8.653 was carried out according to the commonly-used polyethylene glycol method. Next, in order to select the fused cells, culturing was carried out using HAT culture medium in order to select hybridoma cells. After this, in order to confirm whether or not the selected cells produced OCIF-specific antibody, 100 µl of OCIF solution (10 µg/ml) dissolved in 0.1 M sodium bicarbonate solution was added to a 96-well microplate (Nunc Co.) to prepare a solid-phase ELISA, and this was used for measurement of the OCIF-specific antibody in the hybridoma culture solution. The hybridomas observed to show antibody production were cloned repeatedly 3-5 times according to the limiting dilution method, and the antibody production volume according to the above-described ELISA was checked. Those clones showing a large amount of antibody production were selected from among the antibody-producing strains.

ii) Monoclonal antibody production

The antibody-producing strains obtained in Practical Example 25-i) were transplanted into the peritoneum of BALB/c-strain mice inoculated in advance with 1×10^6 Pristan² (Aldrich Chemical Co.). Two weeks after transplantation, the accumulated ascites fluid was collected, and ascites fluid containing the monoclonal antibodies of the present invention was obtained. Purified antibody was obtained from this ascites fluid by means of affinity chromatography using Affigel Protein A Sepharose (manufactured by Biorad Co.). Specifically, the ascites fluid was diluted with an equivalent amount of binding buffer (Biorad Co.) and then run on a protein A column, and it was then washed with an equivalent amount of said buffer. Elution of IgG was carried out using elution buffer (Biorad Co.). After the eluate obtained was dialyzed with water, it was freeze-dried. The purified antibody obtained was subjected to purity testing by SDS-PAGE and found to show a uniform band at a molecular weight position of approximately 150,000.

iii) Selection of monoclonal antibody showing a high affinity for OCIF

The antibodies obtained under Practical Example 25-ii) was dissolved in PBS, and protein determination was then carried out by the Lowry method. Next, the various antibodies were dissolved in PBS to a uniform protein concentration, and the solution was then diluted according to

²Spelling uncertain. - Tr.

the staged dilution method. Using the solid phase ELISA described under Practical Example 25-ii) monoclonal antibodies which reacted with OCIF up to a high dilution stage were selected. As a result, the 3 antibodies A1G5, E3H8, and D2F4 were obtained.

iv) Assaying of antibody subclasses

The classes and subclasses of the antibody of the present invention selected under Practical Example 25-iii) were assayed using an immunoglobulin and subclass analysis kit (Amersham Co.). Testing was carried out according to the protocol specified in the kit. The results are shown in Table 16. E3H8, A1G5, and D2F4 were IgG₁, IgG_{2a}, and IgG_{2b} respectively.

Table 15

Antibody name	IgG ₁	IgG _{2a}	IgG _{2b}	IgG ₃	IgA	IgM	k
A1G5	-	+	-	-	-	-	+
E3H8	+	-	-	-	-	-	+
D2F4	-	-	+	-	-	-	+

v) Method of assaying OCIF by ELISA

The 3 monoclonal antibodies A1G5, E3H8, and D2F4 obtained in Practical Example 25-iv) were solid-phase antibodies and labeled antibodies respectively. Sandwich ELISA was set up by combining them in various ways. Labeling of the antibodies was carried out using a maleimide-activated peroxidase kit (Pierce Co.). The various antibodies were dissolved in 0.1 M sodium bicarbonate solution to a concentration of 10 µg/ml, and they were divided in amounts of 100 µl each into the wells of a 96-well Immunoplate (Nunc Co.) and then left standing overnight at room temperature. Next, the various plates were blocked with a 1/2 concentration of Block Ace (Snow Brand Milk Products Co.) and then washed 3 times with PBS buffer (washing buffer) containing 0.1% Tween 20. The various concentrations of OCIF were prepared with primary reaction buffer (0.2 M tris-hydrochloric acid buffer solution containing a 1/2.5 concentration of Block Ace and 0.1% Tween 20). 100 µl each at various prepared concentrations of OCIF solution was added to the wells, the plate was left standing for 3 hours at 37°C, and it was then washed 3 times with buffer. Dilution of the labeled antibodies was carried out using secondary reaction buffer (0.1 M tris-hydrochloric acid buffer solution containing a 1/4 concentration of Block Ace and 0.1% Tween 20, pH 7.4). The various labeled antibodies were diluted 400 times with secondary reaction buffer and 100 µl of these solutions was added to each of the wells. The plates were then left standing for 2 hours at 37°C, and then after being washed 3 times, 100 µl of a substrate solution (0.1 M citric acid-phosphate buffer containing 0.4 mg/ml of orthophenylene diamine hydrochloride and

0.006% hydrogen peroxide, pH 4.5) was added to the various wells. After the plates were left standing for 15 minutes in a dark room at 37°C, 50 µl of 6 M sulfuric acid was added to the various wells to stop the enzyme reaction, and absorbance was measured at 492 nm using an Immunoreader (NJ2000, Nihon Intermed Co.). The 3 types of antibodies yielded favorable measurement results when used in various combinations of solid-phase antibodies or labeled antibodies, and the 3 types were found to recognize different epitopes of OCIF. As a typical example, Fig. 14 shows a calibration curve with A1G5 as a solid-phase antibody and E3H8 as a labeled antibody.

vi) Assaying of OCIF in human serum

OCIF in the serum of 5 healthy persons was assayed using the ELISA system of Practical Example 25-v) shown in Fig. 14. Specifically, in the same manner as in Practical Example 25-v), A1G5 was solidified on an Immunoplate, 50 µl of the primary reaction buffer was added to the various wells, 50 µl of the various human sera was then added, and the plate was left standing for 3 hours at 37°C. After washing 3 times with washing buffer, 100 µl of E3H8-labeled antibody diluted 400 times with secondary reaction buffer was added to the various wells, and the plate was then left standing for 2 hours at 37°C. After the plate was washed 3 times with washing buffer, 100 µl of the above substrate solution was added to the various wells and a reaction was carried out for 15 minutes at 37°C. 6 N sulfuric acid was added in amounts of 50 µl to the various wells to stop the enzyme reaction, and absorbance was measured at 492 nm using an Immunoreader. The same operation was carried out using primary reaction buffer containing a known amount of OCIF, a calibration curve for OCIF was prepared as shown in Fig. 14, and the amount of OCIF in serum was determined based on the absorbance of the serum samples. The results are shown in Table 16.

Table 16

Serum sample	Amount of OCIF (ng/ml)
1	5.0
2	2.0
3	1.0
4	3.0
5	1.5

[Practical Example 26]

Therapeutic effect on osteoporosis

OCIF was confirmed to have a therapeutic effect in a model of immobility-induced bone atrophy due to nerve excision. Using male Fisher-strain rats, 6-week-old animals (body weight

approximately 120 g) had the nerve plexus of the left upper limb removed in order to induce immobilization of the left upper limb and produce a bone atrophy model. OCIF was prepared with PBS (-) containing 0.01% Tween 80, and beginning on the next day, it was given once daily for a period of 2 consecutive weeks by intravenous administration at 12-hour intervals of dosages of 5 $\mu\text{g/kg}$ and 50 $\mu\text{g/kg}$. Pseudosurgery was carried out in the normal group, and the control group was given administrations of PBS (-) containing 0.01% Tween 80 in the same manner. After completion of administration, the left upper limb was removed and bone strength was measured. The results are shown in Fig. 15.

The results showed that in the control group, there was a decrease in bone strength compared to the normal group, but an improvement was seen in the group administered 50 $\mu\text{g/kg}$ of OCIF.

Possibilities for Industrial Use

The present invention provides a novel protein having an osteoclastogenesis-inhibiting action and an efficient method for its manufacture. The protein of the present invention has osteoclastogenesis-inhibiting activity, and it can be used as a therapeutic agent for various illnesses involving loss of bone mass, such as osteoporosis, or as an antigen, etc., for the immunological diagnosis of such diseases.

Reference to Submitted Microorganisms

Name and address of institution to which microorganisms were submitted

Name: Bioengineering and Industrial Technology Research Institute, Agency of Industrial Science and Technology, Ministry of International Trade and Industry

Address: 1-1-3 Tsukuba-shi, Ibaraki Prefecture, Japan (postal code 305)

Date of submission to institution

June 21, 1995 (original submission date)

Transferred from Bacterial Fermentation Research Deposition No. P-14998 submitted on June 21, 1995, date of submission: October 25, 1995)

Submission Receipt No.: FERM BP-5267

Claims

1. Protein having the following physicochemical properties and an action of inhibiting osteoclast differentiation and/or maturation.
 - (a) Molecular weight (determined by SDS-PAGE): Approx. 60 kD (under reductive conditions), approx. 60 kD and approx. 120 kD (under non-reductive conditions);
 - (b) Affinity: Has affinity for cation-exchangers and heparin;
 - (c) Thermal stability: Activity of inhibiting osteoclast differentiation/maturation decreases due to heat treatment at 70°C for 10 minutes or 56°C for 30 minutes, activity of inhibiting osteoclast differentiation/maturation disappears due to heat treatment at 90°C for 10 minutes;
 - (d) Amino acid sequence: Has as its internal amino acid sequence the amino acid sequences of Sequence Nos. 1-3 in the Sequence Table.
2. Protein according to Claim 1, in which the N terminal sequence is the amino acid sequence shown in Sequence No. 7 of the Sequence Table.
3. Protein according to Claim 1, characterized by producing human fibroblasts.
4. Method for manufacturing the protein according to any of Claims 1-3, characterized in that human fibroblast cells are cultured and the culture solution is purified by carrying out adsorption and elution with an ion-exchange column, an affinity column, and a reverse phase column.
5. Method for manufacturing a protein according to Claim 4, characterized in that cell culturing is carried out using an alumina ceramic piece as a carrier.
6. Protein having the amino acid sequence shown in Sequence No. 4 of the Sequence Table.
7. cDNA coding for the amino acid sequence shown in Sequence No. 4 of the Sequence Table.
8. cDNA shown in the base sequence of Sequence No. 6 of the Sequence Table.
9. DNA, characterized by being hybridized under relatively mild conditions with the cDNA having the base sequence shown in Sequence No. 6 of the Sequence Table.
10. Protein in which cDNA is expressed which codes for the amino acid sequence shown in Sequence No. 4 of the Sequence Table.
11. [page missing] ...showing homology of 80% or more with the amino acid sequence shown in Sequence No. 4 of the Sequence Table.
23. cDNA which codes for the amino acid sequence shown in Sequence No. 13 of the Sequence

Table.

24. cDNA shown by the base sequence of Sequence No. 14 of the Sequence Table.
25. Protein obtained by expressing cDNA shown by the base sequence of Sequence No. 14 of the Sequence Table.
26. cDNA which codes for the amino acid sequence shown in Sequence No. 15 of the Sequence Table.
27. cDNA shown by the base sequence of Sequence No. 83 of the Sequence Table.
28. Protein obtained by expressing cDNA shown by the base sequence of Sequence No. 83 of the Sequence Table.
29. cDNA which codes for the amino acid sequence shown in Sequence No. 62 of the Sequence Table.
30. cDNA shown by the base sequence of Sequence No. 84 of the Sequence Table.
31. Protein obtained by expressing cDNA shown by the base sequence of Sequence No. 84 of the Sequence Table.
32. cDNA which codes for the amino acid sequence shown in Sequence No. 63 of the Sequence Table.
33. cDNA shown by the base sequence of Sequence No. 85 of the Sequence Table.
34. Protein obtained by expressing cDNA shown by the base sequence of Sequence No. 85 of the Sequence Table.
35. cDNA which codes for the amino acid sequence shown in Sequence No. 64 of the Sequence Table.
36. cDNA shown by the base sequence of Sequence No. 86 of the Sequence Table.
37. Protein obtained by expressing cDNA shown by the base sequence of Sequence No. 86 of the Sequence Table.
38. cDNA which codes for the amino acid sequence shown in Sequence No. 65 of the Sequence Table.
39. cDNA shown by the base sequence of Sequence No. 87 of the Sequence Table.
40. Protein obtained by expressing cDNA shown by the base sequence of Sequence No. 87 of the Sequence Table.
41. cDNA which codes for the amino acid sequence shown in Sequence No. 66 of the Sequence Table.
42. cDNA shown by the base sequence of Sequence No. 88 of the Sequence Table.
43. Protein obtained by expressing cDNA shown by the base sequence of Sequence No. 88 of the Sequence Table.

44. cDNA which codes for the amino acid sequence shown in Sequence No. 67 of the Sequence Table.
45. cDNA shown by the base sequence of Sequence No. 89 of the Sequence Table.
46. Protein obtained by expressing cDNA shown by the base sequence of Sequence No. 89 of the Sequence Table.
47. cDNA which codes for the amino acid sequence shown in Sequence No. 68 of the Sequence Table.
48. cDNA shown by the base sequence of Sequence No. 90 of the Sequence Table.
49. Protein obtained by expressing cDNA shown by the base sequence of Sequence No. 90 of the Sequence Table.
50. cDNA which codes for the amino acid sequence shown in Sequence No. 69 of the Sequence Table.
51. cDNA shown by the base sequence of Sequence No. 91 of the Sequence Table.
52. Protein obtained by expressing cDNA shown by the base sequence of Sequence No. 91 of the Sequence Table.
53. cDNA which codes for the amino acid sequence shown in Sequence No. 70 of the Sequence Table.
54. cDNA shown by the base sequence of Sequence No. 92 of the Sequence Table.
55. Protein obtained by expressing cDNA shown by the base sequence of Sequence No. 92 of the Sequence Table.
56. cDNA which codes for the amino acid sequence shown in Sequence No. 71 of the Sequence Table.
57. cDNA shown by the base sequence of Sequence No. 93 of the Sequence Table.
58. Protein obtained by expressing cDNA shown by the base sequence of Sequence No. 93 of the Sequence Table.
59. cDNA which codes for the amino acid sequence shown in Sequence No. 72 of the Sequence Table.
60. cDNA shown by the base sequence of Sequence No. 94 of the Sequence Table.
61. Protein obtained by expressing cDNA shown by the base sequence of Sequence No. 94 of the Sequence Table.
62. cDNA which codes for the amino acid sequence shown in Sequence No. 73 of the Sequence Table.
63. cDNA shown by the base sequence of Sequence No. 95 of the Sequence Table.
64. Protein obtained by expressing cDNA shown by the base sequence of Sequence No. 95 of the

Sequence Table.

65. cDNA which codes for the amino acid sequence shown in Sequence No. 74 of the Sequence Table.
66. cDNA shown by the base sequence of Sequence No. 96 of the Sequence Table.
67. Protein obtained by expressing cDNA shown by the base sequence of Sequence No. 96 of the Sequence Table.
68. cDNA which codes for the amino acid sequence shown in Sequence No. 75 of the Sequence Table.
69. cDNA shown by the base sequence of Sequence No. 97 of the Sequence Table.
70. Protein obtained by expressing cDNA shown by the base sequence of Sequence No. 97 of the Sequence Table.
71. cDNA which codes for the amino acid sequence shown in Sequence No. 76 of the Sequence Table.
72. cDNA shown by the base sequence of Sequence No. 98 of the Sequence Table.
73. Protein obtained by expressing cDNA shown by the base sequence of Sequence No. 98 of the Sequence Table.
74. cDNA which codes for the amino acid sequence shown in Sequence No. 77 of the Sequence Table.
75. cDNA shown by the base sequence of Sequence No. 99 of the Sequence Table.
76. Protein obtained by expressing cDNA shown by the base sequence of Sequence No. 99 of the Sequence Table.
77. cDNA which codes for the amino acid sequence shown in Sequence No. 78 of the Sequence Table.
78. cDNA shown by the base sequence of Sequence No. 100 of the Sequence Table.
79. Protein obtained by expressing cDNA shown by the base sequence of Sequence No. 100 of the Sequence Table.
80. cDNA which codes for the amino acid sequence shown in Sequence No. 79 of the Sequence Table.
81. cDNA shown by the base sequence of Sequence No. 101 of the Sequence Table.
82. Protein obtained by expressing cDNA shown by the base sequence of Sequence No. 101 of the Sequence Table.
83. cDNA which codes for the amino acid sequence shown in Sequence No. 80 of the Sequence Table.
84. cDNA shown by the base sequence of Sequence No. 102 of the Sequence Table.

85. Protein obtained by expressing cDNA shown by the base sequence of Sequence No. 102 of the Sequence Table.
86. cDNA which codes for the amino acid sequence shown in Sequence No. 81 of the Sequence Table.
87. cDNA shown by the base sequence of Sequence No. 103 of the Sequence Table.
88. Protein obtained by expressing cDNA shown by the base sequence of Sequence No. 103 of the Sequence Table.
89. cDNA which codes for the amino acid sequence shown in Sequence No. 82 of the Sequence Table.
90. Genome DNA coding for the amino acid sequence of Sequence No. 4 in the Sequence Table.
91. Genome DNA according to Claim 90, shown by the base sequences of Sequences No. 104 and 105 in the Sequence Table.
92. Antibody showing specific affinity for human osteoclastogenesis inhibitory factor.
93. Antibody according to Claim 92, in which the antibody is a polyclonal antibody.
94. Antibody according to Claim 92, in which the antibody is a monoclonal antibody.
95. Monoclonal antibody according to Claim 95, in which the molecular weight is approximately 150,000 and the subclasses are IgG₁, IgG_{2a}, or IgG_{2b}.
96. Method for assaying human osteoclastogenesis inhibitory factor, characterized by using the antibodies according to Claims 92-95.